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STUDIES ON BACTERIAL PILI

by

LAURA S. FROST

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled STUDIES ON BACTERIAL PILI submitted by LAURA S. FROST in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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For my husband, Edward

ABSTRACT

The purification and preliminary chemical and physical characterization of the F pilus of E. coli and PSA pilus of Pseudomonas aeruginosa K was investigated.

The efficiency of several purification techniques for F pili was studied. Methods involving a precipitation step led to F pilus preparations that were contaminated with protein and carbohydrate material. Moreover, precipitated F pili were found to be extremely insoluble at neutral pH and in standard buffers. Two phase polymer systems were found to be efficient for concentrating F pili from dilute solutions. However, these preparations were also contaminated with cellular debris. F pili could be purified from cells grown on a liquid or solid medium with equal success. Based on a suggestion by Brinton, it was found that F pili could be dissociated from cells and cellular debris in solutions containing 30% sucrose. After removal of the cells by centrifugation and the sucrose by dialysis, F pili were subjected to final purification steps involving differential and isopycnic centrifugation. Approximately 1 - 2 mg of F pili/100 g (wet weight) of cells was obtained using this procedure.

F pili typically banded in CsCl at a density of 1.26 g/cm^3 but strains carrying the F lac plasmid, UBFLO, produced F pili having a buoyant density in CsCl of 1.30 g/cm^3. The F-type R pili produced by cells containing R100-1, R1-19 and R538-1 plasmids were found to have a buoyant density in CsCl of 1.31 g/cm^3. The isoelectric points of F pili from strains carrying two different F plasmids were determined to be pH 3.9 and 4.0. The molecular weight of F pilin is 11,900 as

determined by SDS gel electrophoresis. 1 - 2 molecules of D-glucose/F pilin monomer were detected by paper chromatography of hydrolyzed samples of F pili. F pili were labelled with radioactive ^{32}P sodium phosphate and ^{35}S-methionine. The ^{32}P was found to be covalently attached to the F pilin molecule in a ratio of 2 - 2.5 phosphate groups/F pilin molecule as judged by SDS polyacrylamide gel electrophoresis.

Pseudomonas aeruginosa strain K (PAK) bears polar PSA pili that promote infection by at least six bacteriophages. Moreover, a mutant of strain K (PAK/2PfS) is many times more piliated than the wild-type strain and facilitates the preparation of large amounts of pure pili for biochemical studies.

An investigation into the structural relatedness of PAK and PAK/-2PfS pili and their chemical composition was carried out. A purification procedure is described for PAK and PAK/2PfS pili that yields about 8 mg of pure pili/100 g (wet weight) of PAK/2PfS cells and one-tenth of this amount for PAK cells. PAK and PAK/2PfS pili were found to be free from phosphate, carbohydrate and lipid and to contain a single polypeptide subunit of 17,800 daltons. Isopycnic centrifugation studies revealed that PAK and PAK/2PfS pili have the same buoyant density in sucrose (1.221) and CsCl (1.295). Both types of pili band at pH 3.9 during isoelectric focusing. Amino acid analysis showed that both PAK and PAK/2PfS pili have the same amino acid composition, and microimmunodiffusion studies revealed that the two types of pili are immunologically indistinguishable. It was concluded that PAK and PAK/2PfS pili are identical and that the mutation responsible for producing the multipiliated state in PAK/2PfS is probably located outside the structural gene for PAK pili.

The sequence of the first twenty-two amino acids at the amino terminus was identified using automated sequencing techniques. The first amino acid at the amino terminal was the unusual amino acid N-methylphenylalanine which was identified using gas liquid chromatography, mass spectroscopy and proton magnetic resonance spectroscopy. PAK pili, when treated with carboxypeptidase A, released two amino acids, serine and lysine, from the C-terminus. The conditions for cleavage of pilin with cyanogen bromide were determined and the resultant peptides were separated into three size classes by gel filtration. Preliminary amino acid analysis of these fractions indicated a large fragment of molecular weight 12,000 and smaller fragments of molecular weights 4000 and 1900.

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LIST OF ABBREVIATIONS

F	F transfer plasmid
R	resistance transfer plasmid
Col	Colicin plasmid
Inc	incompatibility
tra	transfer-deficient
fin	fertility inhibition
DNA	deoxyribonucleate
RNA	ribonucleate
EM	electron microscopy
GLC	gas liquid chromatography
MS	mass spectroscopy
PMR	proton magnetic resonance
PEG	polyethylene glycol
SDS	sodium dodecyl sulfate
G	guanosine
PTH	phenylthiohydantoin derivative
DNS	1-dimethylaminonaphthalene sulfonyl derivative
CPA	carboxypeptidase A
CNBr	cyanogen bromide
CTMM	complete tris maleic acid minimal salts medium
Tris	Tris(hydroxymethyl)amino methane
TSB	trypticase soy broth
Ci	Curie; 2.22×10^{12} disintegrations per minute
cpm	radioactive counts per minute
g	centrifugal force relative to gravity

rpm	revolutions per minute
MW	molecular weight
D	dextran
NaDS	sodium dextran sulfate
aa	amino acids
SSC	saline sodium citrate buffer
ρ	density
A_{280}	light absorbance of a solution in a 1 cm light path at 280 nm (for example)
s (following a number)	sedimentation constant in Svedberg units (10^{-13} sec)
m/e	ratio of mass to charge
ppm	parts per million
ε	extinction coefficient of a molar solution
w/v	weight per volume
v/v	volume per volume
V	volts
a (ma)	amps (milliamps)
p	particles
pI	isoelectric pH

All temperatures are expressed in degrees centrigrade.

CHAPTER I

INTRODUCTION

Two types of cellular appendages have been found universally on strains of bacteria: flagella and pili. While the morphology and function of flagella, an organelle of cellular motility, is constant throughout the microbial world (Iino, 1969), the structure and function of pili have been found to vary considerably. Pili can be grossly divided into two categories: somatic pili (Swaney et al., 1977) which are coded for by the bacterial chromosome (Brinton, 1965) and conjugative or donor pili (Novick et al., 1976) which are coded by extrachromosomal elements termed plasmids.

The first plasmid shown to be responsible for the synthesis of pili was the F plasmid (Brinton, Gemski and Carnahan, 1964). The F plasmid had previously been shown to be responsible for bacterial conjugation (Lederberg and Tatum, 1946) where F^+ or Hfr strains of E. coli, i.e. strains with the F plasmid either separate from or integrated into the bacterial chromosome respectively, could transfer genetic material into F^- strains after establishing cell-to-cell contact (Tatum and Lederberg, 1947; Davis, 1950; Hayes, 1952a,b, 1953; Lederberg et al., 1952; Cavalli-Sforza and Lederberg, 1953; Lederberg, 1955, 1956, 1957; Anderson, 1958). Similarly bacterial conjugation was shown to be the mechanism in drug resistance transfer in pathogenic bacteria (Mitsuhashi et al., 1960). In 1964, the presence of F pili on the surface of cells carrying the F plasmid was demonstrated to be the attachment site for a number of donor-specific bacteriophages (Crawford and Gesteland, 1964; Brinton et al., 1964; Brinton, 1965; Valentine and Strand, 1965;

Caro and Schnös, 1966). Other transmissible plasmids such as the drug resistance transfer (R) plasmids were also found to determine the synthesis of pili (Watanabe et al., 1964; Datta et al., 1966; Lawn, 1966). Brinton (1965), and Ou and Anderson (1970) visualized mating pairs of bacteria under a microscope and noted that the cells stayed a fixed distance apart due to the presence of a "bridge" between the cells which they suggested was the F pilus. This requirement for pili during bacterial conjugation was confirmed by Brinton (1965), Novotny et al. (1969b), Curtiss et al. (1969), and Stallions and Curtiss (1972).

Since that time many transmissible plasmids other than F and R plasmids have been found to code for pili. All plasmids have been classified into incompatibility (Inc) groups (Novick et al., 1976) on the basis of two plasmids' inability to coexist in the same cell.

Each "Inc" group has a "type" plasmid to which other plasmids are tested for incompatibility. Many of the "Inc" groups described so far code for pili which are morphologically and serologically unique to that "Inc" group and which are sensitive to different pilus-dependent phages.

Clearly, the role of pili in bacterial conjugation and donor-specific phage infection would be elucidated if more were known about the chemical and physical properties of conjugative pili. However, progress in this direction has been hampered by the small amounts of F pili found in F^+ bacterial cultures; the F plasmid being the best characterized of the plasmids identified to date. Recently Bradley (1977) has found a number of strains of Pseudomonas which produce enough pili to make a thorough chemical characterization including sequencing of the pilus subunit, pilin, possible. These pili have not been demonstrated

unequivocally to be determined by a plasmid nor have they been shown to be involved in bacterial conjugation but they are involved in pilus-dependent bacteriophage infection, and thus seem to have more in common with conjugative pili than type 1 (somatic) pili.

The following is a brief survey of recent reports on studies carried out on pili.

A. THE F PILUS

F-type pili are coded for by three principle types of plasmid: the F plasmid itself, many R plasmids (drug resistance transfer plasmids) and Col plasmids which produce the anti-bacterial agents called colicins and other related bacteriocins (Meynell and Lawn, 1967a, b; Hedges and Datta, 1971, 1972; Dennison, 1972; Hedges, 1972).

The F pilus is a fairly flexible filament of diameter 8.5 - 9.5 nm and 1 - 2 microns long (Brinton, 1965, 1971; Lawn, 1966). Negatively stained pili show an apparent axial hole of 2.5 nm diameter indicating that F pili may be tubular in structure. However Brinton (1971) and Tomoeda et al. (1975) have suggested that the F pilus is composed of two parallel protein filaments on the basis of studies on the controlled disintegration of pili.

The F pilus is composed of a single subunit, pilin, of molecular weight 11,400 to 12,500 (Brinton, 1971; Beard and Connally, 1975; Minkley et al., 1976; Date et al., 1978) which is highly hydrophobic in character and lacks the amino acids histidine, arginine, proline and cysteine (Brinton, 1971; Date et al., 1978). The F pilus is thought to be a phosphoglycoprotein containing one molecule of D-glucose and two molecules of phosphate per subunit (Brinton, 1971). Beard and Connally

(1975) report an additional molecule of glucosamine and galactose in F-type R pilin, and Brinton (personal communication) has reported an extra phosphate group in a mutant of F pili.

The isoelectric point for F pili has been reported as pH 3.8 (Valentine et al., 1969), pH 4.15 (Brinton, 1971), pH 3.5 (Beard et al., 1972b), pH 3.6 (Date et al., 1978) depending on the type of pilus being studied. The buoyant density for F pili in CsCl has been determined to be 1.257 or 1.296 or a combination of both depending on the plasmid and the bacterial host (Brinton, 1971; Beard et al., 1972b; Beard, cited by Tomoeda et al., 1975). Beard reported a buoyant density of 1.31 for F-type R pili and an intermediate density of 1.30 for pili produced by a strain carrying both an F and F-type R plasmid (Beard et al., 1972a).

While F plasmids confer a higher sensitivity to chemical agents upon their host cells (Tomoeda et al., 1975; Goldschmidt and Wyss, 1967; Dowman and Meynell, 1970; Iyobe et al., 1971), the F pilus itself is remarkably resistant to chemical attack. F pili can be digested by trypsin and chymotrypsin (Brinton, 1971) and are dissociated completely in 0.1% SDS or 30 mM Sarkosyl and partially in acid, pH 1.0 or heat at 70°. They show little loss of phage-attachment capability or general structure in reagents such as Brij 58, 8 M urea, 7 M guanidine·HCl or alkali, pH 13 (Date et al., 1978). They are sensitive to organic solvents such as chloroform, carbon tetrachloride or benzene which cause them to dissociate (Brinton and Beer, 1967). This probably reflects the hydrophobic nature of the F pilin molecule.

B. THE GENETICS OF THE F TRANSFER SYSTEM

Four genetically unrelated transfer systems have been identified to

date: F, I, N and P. These systems control the expression of genes coding for pilus synthesis, incompatibility and surface exclusion (the inability of two cells carrying related plasmids to form a mating pair) (Willetts, 1975; Olsen et al., 1974). Only the F transfer system has been studied extensively.

Plasmids in the F transfer system express fertility (now termed transfer) inhibition, Fin^+ (Willetts, 1972; Novick et al., 1976). This involves the ability of any plasmid to repress the expression of the F transfer operon, resulting in a decreased ability of cells to transfer these plasmids or be infected by the F-specific bacteriophages.

A genetic analysis of the F transfer system was reported by Willetts and coworkers (Achtman et al., 1971, 1972; Willetts and Achtman, 1972). Simultaneously Ohtsubo et al. (1970) and Miki et al. (1969, 1970) obtained essentially similar results using different techniques. Using complementation analysis of transfer deficient (tra$^-$) Flac mutants, thirteen transfer genes were identified and subsequently mapped using deletion mutant techniques (Willetts, 1972; Ippen-Ihler et al., 1972). The genetic map of the F plasmid is given in Figure 1 (Willetts, 1975; Achtman et al., 1977).

Briefly, twelve of the tra genes are in the transfer operon and are under the control of the traJ gene product. TraA, traL, traE, traK, traB, traC, traF, traH and part of traG are required for pilus formation, with the traA gene coding for the pilus subunit, pilin (Minkley et al., 1976). TraS and traT mutants are transfer-proficient and produce F pili but lack the property of surface exclusion (Willetts and Maule, 1974, 1977). Recently a protein of molecular weight 25,000 daltons has been found in the outer membrane of F^+ cells (Minkley and Ippen-

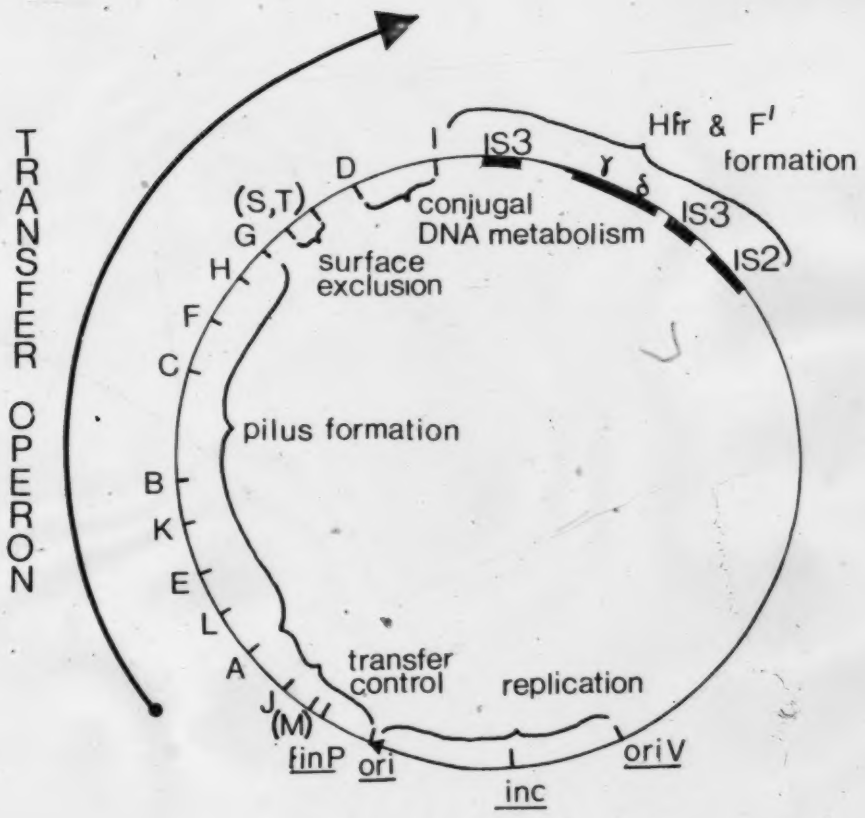

FIGURE 1. The map of the F factor.

The transfer operon (A to I) is indicated by the heavy line drawn clockwise outside the circular map which is not drawn to scale. The cistrons in parentheses have not been as precisely mapped as the other tra cistrons. ori refers to the site of origin of transfer while inc refers to the incompatibility locus. IS1, IS2, IS3 and γδ are insertion sequences. This figure is based on the maps presented by Achtman et al. (1977) and Willetts (1975).

Ihler, 1977; Achtman et al., 1977). This protein, encoded by the traT gene, interferes with mating pair formation. TraS functions independently and reduces DNA transfer (Achtman et al., 1977).

TraD and traI are thought to be concerned with DNA metabolism during transfer and the traI gene is thought to code for a specific endonuclease which initiates DNA transfer (Willetts, 1972, 1975). TraJ is a positive control gene whose product is required for the expression of the transfer operon, traA to traI (Achtman, Willetts and Clark, 1971; Willetts and Achtman, 1972; Finnegan and Willetts, 1973).

Transfer inhibition is due to two structural genes, finO and finP (the finOP system) which control the expression of the traJ gene product and which map closely to the traJ locus (Achtman, Willetts and Clark, 1972; Finnegan and Willetts, 1971; Grindley et al., 1973; Willetts and Dempsey, 1976). The F plasmid is a naturally occurring $finO^-$ mutant which does not exhibit transfer inhibition (Finnegan and Willetts, 1971, 1972; Watanabe and Fukasawa, 1962). Inhibition can be restored if a fin^+ plasmid coexists with the F'plasmid in the same cell. While most F-type plasmids are finOP systems, other systems inhibiting transfer of F-type plasmids but carried by non F-type plasmids have been identified and partially characterized (Grindley and Anderson, 1971; Meynell, 1973; Willetts and Paranchych, 1974; Gasson and Willetts, 1975, 1977).

Most bacterial cultures carrying F-type plasmids are capable of only a very low level of transfer and pilus production because of transfer inhibition (Datta et al., 1966; Egawa and Hirota, 1962; Meynell, Meynell and Datta, 1968; Watanabe, 1963; Willetts, 1970). When a plasmid-free cell receives a $finOP^+$ plasmid, transfer inhibition is

temporarily relieved, giving the phenomenon of HFT (high frequency of transfer) cultures (Watanabe, 1963). This is probably due to transient synthesis of the traJ product before transfer inhibition by the finOP system is established (Willetts, 1974). This phenomenon allows the "infectious spread" of a plasmid throughout the cell population while transfer inhibition eliminates wasteful synthesis of tra proteins, and attack by F-specific bacteriophages (Willetts, 1975).

Small differences in various types of F pili made by different F-type plasmids are reflected in their morphological (Lawn, 1966), serological (Lawn and Meynell, 1970) and F-specific phage adsorbing properties (Dennison and Hedges, 1972; Nishimura et al., 1967). These differences are due to variations in the pilin gene, traA (Alfaro and Willetts, 1972; Willetts, 1971), as are variations in physical (Beard et al., 1972) and chemical (Beard and Connally, 1975) properties. TraA point mutants are capable of transfer but show reduced levels of phage adsorption and plating efficiency (Paranchych, 1975; Willetts, 1975) and thus mimic the naturally occurring F pili variants.

C. THE F PILUS AND CONJUGATION

The first step in conjugation is the formation of a mating pair by the interaction between the tip of the F pilus and a receptor site on the surface of the cell (Reiner, 1974; Skurray et al., 1974). Surface exclusion is thought to prevent mating pair formation by inactivating the receptor site for the tip of the donor pilus (Willetts and Maule, 1974; Achtman et al., 1977). Pre-treatment with pilus tip-specific agents such as filamentous DNA phages (Ippen and Valentine, 1967; Novotny et al., 1968; Ou, 1973) or Zn^{++} ions (Ou and Anderson, 1972) blocks

conjugation. Using a mechanism not clearly understood, a signal is transmitted along the F pilus to trigger the synthesis or activation in the donor cell of the enzyme(s) required for DNA metabolism during transfer. The DNA is nicked at a specific site (see above) and a specific strand of DNA (Vapnek and Rupp, 1970) is transferred, 5'-terminal first, into the recipient cell (Ohki and Tomizawa, 1968). The F plasmid, when integrated into the host chromosome (HFr strains), causes the transfer of all or part of the chromosome as well. Transfer is accompanied by replication and circularization of this strand in the recipient cell as well as of the remaining strand in the donor cell (Vapnek et al., 1971; Vapnek and Rupp, 1970). Whether the DNA strand proceeds via the F pilus into the recipient cell or whether the F pilus merely draws the cells together to form a classical conjugation bridge is under much dispute (Brinton, 1971; Paranchych, 1975; Tomoeda et al., 1975).

D. F-SPECIFIC PHAGE INFECTION

In addition to their role in bacterial conjugation, F pili also function as receptors for F-specific phages. Two types of F-specific bacteriophages have been identified: the spherical RNA phages which attach to the sides of the pilus (Crawford and Gesteland, 1964; Brinton et al., 1964), and the filamentous DNA phages which attach to the tip of the pilus (Caro and Schnös, 1966).

The phages which attach to the sides of F pili are simply constructed icosahedral virions, approximately 26 nm in diameter, containing a single-stranded RNA molecule surrounded by 180 identical coat proteins (Vasquez et al., 1966). A single attachment (A) protein is exposed on

the surface of the virion (Curtiss and Krueger, 1974) and is noncovalently attached to the phage RNA (Krahn et al., 1972), probably at the 3'-terminal end (Wong and Paranchych, 1976b). Typical RNA phages include R17, MS2, M12, F2 and Qβ.

The early stages of the RNA phage infectious process involve the attachment of the phage particle via the A-protein to the side of an F pilus followed by a pilus-mediated cleavage of the A-protein into two peptides, ejection of the A-protein - RNA complex from the virion, and transfer of this complex to the surface of the cell where it penetrates into the cell interior (Paranchych, 1975).

The attachment of the phage to the F pilus is the function of the A-protein (Argetsinger and Gussin, 1966; Steitz, 1968b). This attachment step can occur with cell-associated or cell-free pili (Crawford and Gesteland, 1964; Brinton et al., 1964; Wendt et al., 1966), is reversible (Paranchych et al., 1970), diffusion-limited with no energy of activation (Knolle, 1967a) and requires an ionic strength of 0.1 or more (Danziger and Paranchych, 1970a).

When the A-protein - RNA complex is ejected from the virion, the empty phage capsid is released from the F pilus (Paranchych, 1966; Silverman and Valentine, 1969). It has been suggested that the F-pilus triggers the ejection step by catalyzing the cleavage of the A-protein into two peptides (Krahn et al., 1972). This step is energy-requiring (Knolle, 1967b) and therefore occurs only with cell-associated pili. While a large number of RNA phages can attach to a bacterium's F pili, a relatively small number penetrate the cell (Paranchych, 1975). This may be due to a steric inhibition of pilus retraction by large numbers of phage particles adsorbed to the pilus surface.

The filamentous DNA phages, such as f1 and M13, attach to the tip of the F pilus. These bacteriophages consist of a circular, single-stranded DNA molecule packed into a viral capsid 5.5 nm in diameter with a modal length of 0.7 - 0.9 μ (Marvin and Hohn, 1969) and contain an attachment protein at one end (Marvin, 1966).

The early stages of DNA phage infection closely follow that for RNA phages but a few differences have been noted. The attachment process is irreversible (Tzagoloff and Pratt, 1964) and the entire virion is thought to penetrate the cell surface resulting in the absence of a DNase-sensitive step (Tzagoloff and Pratt, 1964; Trenkner et al., 1967; Smilowitz, 1974).

E. POSSIBLE MECHANISMS FOR PILUS-MEDIATED TRANSFER OF NUCLEATES

The mechanism by which the F pilus mediates the transfer of plasmid DNA during conjugation or carries viral RNA or DNA to the cell surface is as yet unclear. Two principal models exist: one based on the F pilus remaining extended from the cell surface and actually transporting the nucleic acid itself, and one in which the F pilus is not involved in nucleic acid transport directly but retracts, bringing the recipient cell or phage particle into close contact with the donor cell.

The first model has been proposed by Brinton (1965, 1971) and consists of two variations. The first variation envisions the F pilus to be a hollow tube of 25 nm inner diameter through which the nucleic acid passes. This model has been largely discredited because no DNA - F pilus complex has ever been detected (Tomoeda et al., 1975). Moreover, since phage RNA retains at least 50% of its secondary structure during its penetration into the cell (Wong and Paranchych, 1976a), it has been

concluded that the RNA must remain on the exterior surface of the pilus. The other variation (Brinton, 1971) states that the F pilus is composed of a pair of filaments which transfer the plasmid DNA or phage particle by conduction (transfer occurs in the groove between the filaments), by conveyor belt (the two filaments move with respect to each other, in conveyor belt fashion) or by a carrier mechanism (the pilus is assembled in the donor cell membrane and depolymerized in the recipient cell membrane). The best evidence for these models is the visualization in the light microscope of mating pairs of E. coli staying a fixed distance apart, presumably connected by an F pilus (Brinton, 1965; Ou and Anderson, 1970).

Pilus retraction was first proposed by Marvin and Hohn (1969). Jacobsen (1972) and Bradley (1972b,c,d, 1973a,b, 1974b) have shown that average pilus length decreases with time after infection of E. coli or P. aeruginosa cells with pilus-dependent bacteriophages and that the phage capsids accumulate at the cell surface.

Novotny et al. (1972) reported that F pili rapidly disappear from the cell surface when cyanide is added to bacterial cultures. Similarly, O'Callaghan et al. (1973c) found that F pili disappeared from surfaces of glycerol-grown cultures when treated with arsenate and Novotny and Lavin (1971) noted F pili disappearance when the temperature of a bacterial culture was suddenly dropped to 20°. Novotny and Fives-Taylor (1974) and Bradley (1972c,d) found that pilus retraction was prevented by treatment with pilus-antiserum or RNA phages as seen by the cell's inability to undergo filamentous DNA phage infection or conjugation. Multi-piliated strains of E. coli (Moore and Paranchych, unpublished results) and P. aeruginosa (Bradley, 1972c, 1974) are

resistant to pilus-dependent phages and do not lose their pili at low temperatures indicating a loss of ability to retract their pili.

It seems feasible that the above observations indicate an equilibrium exists between pilus formation and outgrowth and pilus retraction (Bradley, 1972b; O'Callaghan et al., 1973b) and that this equilibrium is disturbed in favor of pilus retraction by various poisons, by temperature shifts and by the attachment of pilus-specific objects (antiserum, phage, recipient cells). This equilibrium may be shifted toward enhanced pilus outgrowth by treatments such as the incorporation of 5-bromouracil into DNA using an F transfer system (Fives-Taylor and Novotny, 1974) or by the addition of I-type pilus antiserum to cells carrying an I-type R plasmid (Lawn and Meynell, 1972).

Studies on the reappearance of donor pili on cells depiliated by various techniques (Novotny et al., 1969a) indicate that a pool of pilin subunits exists in the cell membrane which is of a constant concentration of about twice the amount of mature, assembled, pilin molecules and can restore full piliation in 4 to 5 minutes (Novotny et al., 1972; Brinton, 1965, 1971). Beard and Connally (1974) have reported finding a pool (7×10^4 molecules) of F-type pilin molecules in the outer membrane of E. coli cells carrying a derepressed F-like R factor.

These results show that pilus outgrowth does not require de novo protein synthesis but requires energy (perhaps in a phosphorylation and/or glucosylation step) and is balanced by pilus retraction, the mechanism of which is unknown. The accumulation of free pili in the culture medium may not be as general a phenomenon as has previously been thought (Brinton, 1965, 1971; Novotny et al., 1969a).

Whether the transfer of genetic material by F pili occurs by

conduction or retraction or a combination of both is still uncertain. The apparent simplicity of the F pilus structure suggests a single mechanism for the transfer of single-stranded nucleic acids either towards or away from the host cell (Paranchych, 1975).

F. F PILUS PURIFICATION

Three principal methods are generally used to estimate relative amounts of pili. The electron microscope is excellent for studying pilus morphology, determining size and number of pili per cell, and identifying large contaminants such as flagella and type 1 pili. A second method is based on the assay of the amount of radioactive-labelled phage adsorbed to F pili. The filtration assay procedure (Danziger and Paranchych, 1970a), in which radioactive phage-pilus complexes are caught on membrane filters, measures both cell-associated and cell-free pili but is not quantitative since a certain fraction of these complexes is washed through the filter (Brinton and Beer, 1967). A variation on this assay technique measures cell-associated pili by saturating a cell's pili with radioactive phage, centrifuging out the cells, and quantitating the amount of radioactive phage in the cell pellet (Paranchych, 1975). A third method (Novotny and Lavin, 1971) gives an accurate estimation for the amount of F pili in a culture relative to a second, standard, culture. It depends on the ability of F-type pilus antiserum to bind specifically to the F pilus and interfere with DNA phage infection (Ishibashi, 1967; Lawn et al., 1967). Thus a standard curve relating plaque-forming units (PFU) to cell concentrations before and after the addition of the antiserum indicates the amount of cell-associated F pili in the culture. The work reported in this thesis

uses only the first two methods described above.

Purification procedures reported for F pili are similar to those employed for the purification of various viruses. The removal of F pili from the cell surface has been accomplished by blending (Brinton, 1965; Valentine and Strand, 1965; Novotny et al., 1969a), or by the more gentle stirring of cells in 30% sucrose with a magnetic stirring bar (Brinton, 1971; Minkley et al., 1976). The presence of sucrose dissolved the pili, and prevented non-specific binding of free pili to cells (Brinton, 1971). Brinton (1971) and Minkley et al. (1976) achieved a yield of 5 mg of F pili from a slightly multi-piliated mutant Flac strain, from 24 litres of culture at 95% purity by processing the sucrose-dissolved pili solution in the following manner. The cells were removed by centrifugation and the supernatant was extensively dialyzed to remove sucrose. The removal of sucrose caused the pili to aggregate into large "crystals" which could be easily centrifuged out of solution. The concentrated pili were banded in a CsCl buoyant density gradient as a final purification step. Minkley et al. (1976) reported that the pili of a less well-piliated mutant were more difficult to purify and required solubilization of the pili in SDS followed by Biogel P150 column chromatography to achieve pure pilin.

Recently Date et al. (1978) have purified F pili using Brinton's method but included a sucrose gradient sedimentation step in 0.1 M NaCl to separate F pili from contaminants of low density. They claimed that 1.5 - 2.0 mg of pure F pili could be obtained from 67 g (wet weight) of cells.

Beard et al. (1972) published a purification procedure for F-type

R pili which consisted of four stages: (1) blending the culture and removing the cells by centrifugation; (2) ultrafiltration to concentrate the pili; (3) CsCl density gradient centrifugation; (4) isoelectric focusing to separate the conjugative pili from type 1 pili. They claimed 95% purity and a yield of 3 - 6 mg of protein from 20 litres of culture.

Other methods, which result in crude F pili preparations, include precipitation at the isoelectric point of F pili (pH 4.0) by the addition of HCl (Brinton, 1965; Brinton and Beer, 1967; Valentine et al., 1969) and precipitation with polyethylene glycol and NaCl (Tomoeda et al., 1975).

While the F plasmid and the F transfer system have yielded much information on bacteriophage infectious processes and bacterial conjugation, it may not prove to be the best system for studying the chemistry of pili since so little material is produced in cells carrying the F plasmid. Frost and Paranchych (1977) calculated the theoretical yield of F pilin from a culture, bearing one pilus per cell, to be 1 mg in 1000 litres. Since this was clearly impractical, attempts were made to find one or more multi-piliated bacterial strains.

G. THE PSA PILI OF PSEUDOMONAS AERUGINOSA

The most promising source of multipiliated bacteria appeared to be various strains of Pseudomonas aeruginosa carrying polar (PSA) pili (Bradley, 1966).

PSA pili produced by various P. aeruginosa strains are of similar appearance, i.e., they are long, flexible filaments with no visible subunit structure, of dimensions 6 nm in diameter and a modal length of

2,500 nm (Bradley, 1972a; Weiss, 1971), but they are usually serologically unrelated (Bradley and Pitt, 1975). The origin of these pili is unknown; attempts to correlate them with the presence of the FP2 or FP39 sex factors of Pseudomonas aeruginosa (Inc P-8) have failed (Holloway, 1969). If they are coded for by a nonconjugative plasmid, it can not be mobilized by the P-1 type plasmid RP1 (Bradley and Pitt, 1975).

PSA pili are required in the infectious process of three different types of phages: (1) spherical RNA phages (Bradley, 1966; Feary et al., 1964); (2) filamentous DNA phages (Takeya and Amako, 1966); (3) spherical DNA phages with non-contractile tails (Bradley, 1973b; Bradley and Pitt, 1974).

The RNA phage, PP7 (Bradley, 1966; Weppelman and Brinton, 1971), is similar to the F-specific RNA phages of E. coli. PP7 is specific for Pseudomonas aeruginosa strain PA01. It attaches to the pilus irreversibly at pH 8.4 at a ratio of 1 phage/pilus but the attachment is less specific at lower pH's (Bradley, 1976). No RNA phage has been found for strain K (Bradley, 1974b). PP7 cannot saturate the PSA pilus with many closely-aligned phage particles as is the case with the F-specific RNA phages.

The filamentous DNA phage, Pf, which resembles the F-specific DNA phages, attaches to the tip of PSA pili of strain K (Bradley, 1973a). The DNA phages with spherical heads and non-contractile tails of varying lengths (Bradley, 1973b; Bradley and Pitt, 1974) have no counterpart in strains of E. coli carrying F-type plasmids. They attach to the sides of the PSA pilus by means of lateral tail fibers.

Bradley (1972a,b,c,d; 1973a,b; 1974b) has shown that attachment of

these three classes of phage is followed by pilus retraction and the transport of the phage particle to the cell surface where ejection and penetration of the phage genome occurs. A phage receptor site in the outer membrane for the tailed bacteriophages has been postulated by Bradley (1972c, 1974).

An unexpected bonus from these experiments was the isolation of multi-piliated mutants of strains PA01 and K, named PA068 and K/2PfS respectively (Bradley, 1972a,b; 1974), which were resistant to PSA pilus-dependent phage infection. These strains could attach phage particles but could not retract their pili. K/2PfS was the more-piliated of the two mutants, producing 50 to 100 pili per pole. The wild-type strain, K, produced ten to twenty retractile pili per pole. Besides flagella, these strains produced no other potentially contaminating types of pili such as type 1 pili.

H. THE PILI OF OTHER INCOMPATIBILITY GROUPS

While pili are encoded by plasmids of at least six other incompatibility groups besides F, comparatively little is known about them at present, and in any event, they are not pertinent to the results discussed in this thesis. A brief description of their morphology and function is given in Table 1.

The Inc I group comprising many I-type R and Col plasmids produces I pili which are morphologically and serologically distinct from F pili (Lawn and Meynell, 1970; Lawn et al., 1967) and adsorb the I-specific filamentous DNA phages, such as If1 (Meynell and Lawn, 1968). No I-specific RNA phages have as yet been isolated. The I transfer system parallels the F transfer system with respect to transfer initiation

TABLE 1

A Comparison of the Morphology and Phage-Sensitivities of the Types of Pili Reported to Date

Inc Group	Type Plasmid	Pilus Type	Dimensions (nm)	Morphology	Phage Sensitivity	Reference
FI	F	F	9 x 1500	flexible, 2-3 filaments/pilus	$f1^{S}R17^{S}Q\beta^{S}$	(Brinton, 1971)
FV	$F_o$lac	$F_o$lac	9 x 1500	"	$f1^{S}R17^{R}Q\beta^{R}$	(Falkow and Baron, 1962)
I	Col I	I	7 x 1000	flexible, non-polar	If1, If2	(Lawn, 1966)
T		T				(To et al., 1975)
W	Sa	W	12 x 450	flexible, non-polar, pointed tip	PRD-1	(Bradley,1977a)
P-1	RP1	P-1	8 x 300	rigid, mainly polar	PRR1, Pf3, PRD-1	(Bradley, 1977a)
P-2	R931	P-2	n.d.	small, flexible non-polar	$R17^{R}If1^{R}$, $PP7^{R}$, $PR5^{R}$	(Shahrabadi et al., 1975)
X	R6K	X(711b)	9.5 x 1200	flexible, non-polar	$f1^{S}R17^{R}Q\beta^{R}$	(Bradley, 1977b)
X		X(485)	5 x 260	flexible, helical non-polar	n.d.	
N	N3	n.d.	-	-	IKe, PRD-1	(Khatoon et al., 1972)
		PSA	6 x 2500	rigid, polar	PP7, Pf, P04	(Bradley, 1972a)
		type 1	7 x 1000	rigid, helical	n.a.	(Brinton, 1965)

n.d. not determined; n.a. not applicable

(Fenwick and Curtiss, 1973a,b), DNA strand specificity (Vapnek et al., 1971) and DNA metabolism (Fenwick and Curtiss, 1973a,b).

I-type plasmids exhibit the properties of surface exclusion (Fenwick and Curtiss, 1973b) and transfer inhibition in a two-step control system analogous to the finOP system of F-type plasmids (Meynell et al., 1968; Ohki and Ozeki, 1968; Ozeki, 1965). The F and I systems do not complement one another (Lawn et al., 1967; Romero and Meynell, 1969).

The R plasmids found in Pseudomonas have been classified into incompatibility groups P-1, P-2 etc. on the basis of their incompatibility with one another in Pseudomonas (Shahrabadi et al., 1975).

Inc P-1 group plasmids code for pili (Bradley, 1974b) and a surface exclusion system (Hedges and Jacob, 1974). They do not express fertility inhibition but can be inhibited by plasmids from other incompatibility groups (Jacoby, 1977).

The group plasmids are sensitive to three types of pilus-dependent phage: the spherical RNA phage PRR1 (Olsen and Thomas , 1973); the filamentous DNA phage Pf3 (Stanisich , 1974); and the lipid-containing DNA phages such as PRD1, which attach to the pilus tip (Olsen et al., 1974), and have a contractile tail which is not used in pilus attachment (Bradley, 1977a).

P-1 pili are short and rigid and generally occur at the poles of the cell (Bradley, 1977a) and undergo retraction during P-1 - specific phage infection (Bradley, 1977a). No transfer inhibition system has been identified for P-1 plasmids (Chandler and Krishnapillai, 1974; Olsen et al., 1974; Olsen and Shipley, 1973).

Inc P-2 group plasmids, however, do have a transfer inhibition system which has made study of their short non-polar pili difficult (Shahrabadi et al., 1975).

W pili (Bradley, 1975b; Bradley and Cohen, 1975) produced by the Inc W group plasmids, such as Sa, are short, pointed, non-polar pili with the unusual optimum growth temperature of 30° in stationary phase cultures. They have been implicated in conjugation of W plasmids (Bradley, and Cohen, 1976) attach the lipid-containing DNA phages, such as PRD-1, to their tip and undergo retraction (Bradley, 1977a).

Inc N group plasmids, which have no transfer inhibition system but can inhibit co-existing P-1 plasmids (Chandler and Krishnapillai, 1974; Stanisisch, 1974), can in turn be inhibited by the Inc X plasmid R6K (Pinney and Smith, 1974). Inc N plasmids confer sensitivity to the DNA phage, IKe, (Khatoon et al., 1972) and lipid-containing phages such as PRD-1 (Bradley, 1977a), but no pili have yet been shown to be encoded by these plasmids (Bradley, 1977a). Recently a somatic receptor site for PRD-1 phage has been characterized as a pool of proteins (molecular mass, 51,000 daltons) in the outer membrane of PRD-1 sensitive cells (Olsen et al., 1977). They postulated that this was a pool of pilin molecules analogous to the F-pilin molecules found in the outer membrane of E. coli by Beard and Connally (1975).

Also, To et al. (1975) have reported a temperature sensitive pilus coded for by Inc T group plasmids but no detailed study has been published.

The Inc X group of plasmids has been shown to specify two types of pili. The F plasmids R711b and R778b (Hedges, 1974; Bradley, 1977b) code for F-like pili which confer sensitivity to the F-specific filamentous DNA phages, i.e., f1, but not the F-specific RNA phages, and therefore resemble the pili produced by the $F_o$lac plasmid of Inc FV (Datta, 1975; Falkow and Baron, 1962). The plasmid R485 specifies very

thin pili with an obvibusly helical character as seen by electron microscopy. No phage specific for R485 pili has yet been isolated (Bradley, 1977b).

I. TYPE I PILI OF ESCHERICHIA COLI

Type I pili are the prototype of somatic pili in E. coli. They were first characterized from strains of E. coli B but are also found on the cells of strains of E. coli K12 either maintained in laboratory cultures or isolated from clinical sources. Type I pili have a diameter of about 7 nm and typical lengths of 0.5 to 2 microns. They are found in large numbers over the surface of the cell and are self-assembling helices of identical protein subunits (MW = 17,000) which do not contain phosphate or carbohydrate. They may be concentrated and purified by crystallization in the presence of magnesium ion. Type I pili are coded for by the bacterial chromosome and do not have a role in conjugation nor have they been shown to be involved in pilus-dependent phage infection (Brinton, 1965). One of their functions is adhesion to mammalian cells and to other surfaces where attachment is usually end-wise (Duguid et al., 1966).

Recently they have been implicated in contributing to the virulence of enteropathogenic strains of E. coli which cause series disease in man and animals (Swaney et al., 1977).

Some of the strains in this study produce type I pili which was one of the principal sorts of contamination in F pili preparations.

The present program was originally directed towards purification of F pili from a wide range of naturally-occurring or genetically-induced pilus mutants which produce F pili but are resistant to certain classes of F-specific bacteriophages. By comparing the chemical composition of these pili, it was hoped that insight into the number and kinds of reactive sites in the F-pilin molecule could be obtained. However, the amount of F pili produced by bacterial cultures carrying these plasmids was extremely low, making the prospect of being able to perform an adequate characterization of these proteins rather poor. For this reason, the wild-type and multi-piliated mutants of Pseudomonas aeruginosa K were used as a source of pure pili to acquaint ourselves with the problems and techniques in the purification and chemical characterization of pili in general. This system was both promising and interesting, for not only were these pili involved in the infectious process of several bacteriophages, and capable of retraction, they were also present in large numbers on the cell. In addition, two strains, the wild-type, PAK, and the multi-piliated mutant, PAK/2PfS, were available. Thus, a comparison of the structural relatedness of PAK and PAK/2PfS pili would indicate whether or not the mutation responsible for the loss of pilus-retraction in PAK/2PfS was in any way reflected in their biochemical composition, and provide a basis of comparison of PAK and other types of pili.

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. Bacteria and bacteriophage

(a) Bacteriophage

The RNA phage, R17, isolated by Paranchych and Graham (1962), was used throughout these studies.

(b) Bacteria

The various strains of *Escherichia coli* and *Pseudomonas aeruginosa* used in the course of these studies are given in Table 2.

Except where specified, WP156 was used for preparing R17 phage lysates.

All strains were preserved for long periods of time by lyophilizing a small portion of a culture grown in 5% glucose, 5% peptone, pH 7.2 and sealing the tube while under vacuum. Bacterial strains were resuscitated by resuspending the lyophilized cells in 0.5 ml of broth and streaking these cells out on hard agar slants in 5.0 ml Universal bottles to maintain the strain for several months. The cultures grown on the slants at 37° were used to streak hard agar for routine use. Cell cultures were started by transferring a single colony on an agar plate to 10 ml of liquid medium which was grown at 37° with or without shaking overnight (16 h).

2. Bacterial culture media

(a) TMM medium (Tris (hydroxymethyl) amino methane maleic acid minimal salts medium)

The basic TMM salts solution contained the following components:

TABLE 2

Bacterial Strains

Designation	Strain		Source/Derivation
1. HB11 B/r (HB11)	*E. coli* B	F'lac (HBFLO)/lac fla pil	C.C. Brinton
2. HB11 B/r F^- (HB11 F^-)	*E. coli* B	F^- lac fla pil	HB11 treated with acridine orange
3. JC3272	*E. coli* K12	F^- lac his trp str^r spc^r $T_6{}^r$ gal lys	N.S. Willetts
4. JC6449	*E. coli* K12	F'lac (JCFL*traD60*)/JC3272	N.S. Willetts
5. ED2601	*E. coli* K12	F^- lac his trp str^r spc^r $T_6{}^r$ gal lys fla	W. Paranchych
6. ED2602	*E. coli* K12	F'lac (JCFLO)/ED2601	W. Paranchych
7. ED2603	*E. coli* K12	R100-1 ($R17^r$ $M13^s$)/ED2601	W. Paranchych
8. ED2604	*E. coli* K12	R1drd19 ($R17^s$ $M13^s$)/ED2601	W. Paranchych
9. ED2612	*E. coli* K12	F'lac (JCFL*traD8*)/ED2601	W. Paranchych
10. ED2687	*E. coli* B	F'lac (JCFLO)/HB11 F^-	W. Paranchych
11. ED2692	*E. coli* B	F'lac (JCFL*traD8*)/HB11 F^-	W. Paranchych
12. WP128	*E. coli* B	R538drd1/HB11 F^-	W. Paranchych

continued...

Table 2 - continued

Designation	Strain		Source/Derivation
13. UB1105	E. coli K12	F'lac (UBFLO)/lac leu fla str^r	J.P. Beard
14. WP156	E. coli K12	Hfr met T_1^r str^s	P. Krahn
15. PAK	P. aeruginosa K	pil	D.E. Bradley
16. PAK/2PfS	P. aeruginosa K	pil Pf^r (small colonies)	D.E. Bradley

Tris, 0.005 M; maleic acid, 0.05 M; 0.043 M NaCl; 0.027 M KCl; 0.019 M NH_4CL; 1 mM Na_2HPO_4; 1 mM NaH_2PO_4.

The above compounds were dissolved in distilled, deionized water, the pH was adjusted to pH 7.3 and the solution was autoclaved at 126° for 15 min under a steam pressure of 20 lb/in^2.

(b) CTMM (Complete TMM)

Complete TMM (CTMM) medium was prepared by combining the following sterile components listed in the following proportions:

TMM	91 parts
Eagles' amino acid concentrate (100 X) (Difco) *	1 part
50% (w/v) glucose	1 part
0.25% (w/v) L-methionine	1 part
0.5 M $MgCl_2$	1 part
0.020% d-biotin	5 parts

Various TMM-based media included:

(i) lac CTMM. This medium included 2 parts 25% (w/v) lactose instead of glucose.

(ii) CTMM-aa. This medium contained the regular components of CTMM with the omission of the Eagles' amino acid concentrate.

(iii) CTMM + casamino acids. Casamino acids (Nutritional Biochemicals Co., 0.05%) replaced the Eagles' amino acid mixture.

(iv) Low phosphate CTMM. The concentration of Na_2HPO_4 in the TMM

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*Eagles' amino acid concentration 100 X (Difco) contained the following (per ml): arginine, 1.7 mg; cystine,=1.2 mg; tyrosine, 1.8 mg; histidine, 0.8 mg; isoleucine, 2.6 ml; leucine, 2.6 mg; lysine, 2.6 mg; methionine 0.75 mg; phenylalanine, 1.7 mg; threonine, 2.4 mg; tryptophan, 0.4 mg; valine, 2.4 mg; biotin, 0.1 mg; folic acid, 0.1 mg; choline chloride, 0.1 mg; nicotinamide, 1 mg; Ca pantothenate, 0.1 mg; pyridoxal hydrochloride, 0.1 mg; thiamine hydrochloride, 0.1 mg; riboflavin, 0.01 mg; inositol, 0.18 mg; phenol red, 0.5 mg.

minimal salts solution was reduced to 0.1 mM.

(c) Trypticase soy broth (TSB)

TSB (Baltimore Biological Laboratories [BBL])	15 g/l
NaCl	8 g/l

Autoclaved solutions had a final pH of 7.2 - 7.3.

(d) Top agar

Trypticase soy broth	30 g/l
Bacto-agar (Difco)	11 g/l

Sterile top agar was stored in 50 ml volumes at 4° until used. For plaque assays or viable cell counts, the agar was melted in a boiling water bath and dispensed into sterile culture tubes in 1.5 ml aliquots maintained at a temperature of 50° in a water bath.

(e) Hard agar (TSB agar)

Trypticase soy broth	30 g/l
Bacto-agar	15 g/l

After dissolving, the solution was autoclaved and dispensed into petri dishes while warm.

3. Buffers

(a) Bacteria and phage diluent

All dilutions of bacteria or phage were made with a sterile solution composed of:

0.9% (w/v) NaCl

5 mM $MgCl_2$

5 mg % bovine serum albumin (Sigma Chemical Co.)

$MgCl_2$ was autoclaved separately and added to the sterile 0.9% NaCl. The albumin was supplied as a sterile solution and was used directly. Diluent was dispensed in 10 ml volumes into sterile 20 mm

dilution tubes and stored at 4°. These solutions were warmed to room temperature before use.

(b) TMM + Mg^{++}

Sterile 5 mM $MgCl_2$ was added to the basic minimal salts solution (TMM) which had previously been autoclaved.

(c) Saline sodium citrate buffer (SSC)

0.15 M NaCl	8.7 g
0.015 M Na citrate	4.41 g/l

The desired pH was obtained by the addition of concentrated NaOH.

4. Chemicals, enzymes and reagents

All reagents were prepared from analytical-grade chemicals in double-distilled water unless otherwise specified. Ultrapure sucrose and dithiothreitol were used as supplied by Schwarz/Mann. CsCl was obtained from Gallard-Schlesinger. Ampholytes were obtained from LKB Produktes, Bromma, Sweden. Sodium dodecyl sulfate (SDS), technical grade, was obtained from Matheson, Coleman and Bell. Sodium phosphate, both mono- and dibasic, was obtained from Mallinckrodt Chemical Works.

Chemicals used for polyacrylamide gel electrophoresis were obtained from Eastman Organic Chemical Co. Acrylamide was recrystallized from acetone. Myoglobin, chymotrypsinogen and lysozyme were obtained from Schwarz/Mann. Carbonic anhydrase and DFP-treated carboxypeptidase A were obtained from Worthington Biochemicals Corp.

5. Radioactive materials

Radioactive precursors for the preparation of labelled F pili and R17 phage were obtained from the following sources: ^{32}P, ^{33}P (as $H_3{}^{32}PO_4$ or $H_3{}^{33}PO_4$, carrier free, in 0.02 M HCl); ^{35}S (as $Na_2{}^{35}SO_4$

in water, 800 mCi/mmole) and ^{3}H-L-amino acid mixture (5.0 mCi in 0.322 mg 0.1 M HCl) from New England Nuclear; ^{3}H-8-guanosine (>5 Ci/mmole) from Amersham/Searle.

B. GROWTH OF BACTERIA

Bacterial cultures in liquid media were grown at 37° in a rotary-shaking water bath (New Brunswick Scientific Co.) from a 1:100 dilution of an overnight culture grown in an identical medium. Maximum aeration and minimum pilus breakage was achieved by using shallow cultures, generally one fifth of the flask volume, shaken at 125 rpm in baffled culture flasks (Bellco Glass Co.). Under these conditions, cultures generally reach a cell density of 5 x 10^{8} cells/ml in 4 hours using minimal media and 2 - 2.5 hours for rich media. The density of E. coli cells in various media was determined from a standard curve constructed by plotting the absorbance at 650 nm of a 1.0 ml volume of culture in a cuvette (with a light path of 1 cm) vs. the viable cell count. Viable cell counts were determined by plating 1.0 ml of an appropriate dilution of the culture mixed with 1.5 ml soft top agar which was spread on TSB hard agar plates. The plates were incubated overnight at 37° and scored for bacterial colonies.

C. PREPARATION AND PURIFICATION OF PHAGE R17

A culture of WP 156 cells, grown in CTMM-aa at 37° to a cell density of 4 x 10^{8} cells/ml, was infected with purified R17 phage at a multiplicity of 40 PFU's/cell. The infected culture was allowed to lyse with vigorous shaking (150 rpm) for 3-4 hours at which time cell lysis was completed by adding lysozyme (66 mg/l) and chloroform

(3.3 ml/l) during the last 30 min of the incubation. The cell debris was removed by low speed centrifugation and the resultant crude lysate was titered using the plaque assay method. This method consists of plating 1.0 ml of an appropriate dilution of the phage solution to which 0.2 ml of bacteria (WP156 cells at 4 x 10^8 cells/ml) and 1.5 ml of molten agar has been added. The mixture is spread on a TSB hard agar plate and incubated overnight at 37°. Typically 8 x 10^{11} to 1.2 x 10^{12} PFU/ml were obtained in a crude lysate. The phage was purified from the crude lysate before the removal of the cellular debris by a liquid two-phase polymer system (Albertsson, 1960) containing 0.21 g sodium dextran sulfate, 7.15 g polyethylene glycol 6000 and 1.8 g NaCl per 100 ml of crude lysate. The R17 phage was extracted from the sodium dextran sulfate phase and was banded in an equilibrium CsCl density gradient at 1.44 g/cm^3 using 2.4 g CsCl/4.0 ml of R17 phage solution in SSC buffer.

The number of phage particles in a phage solution was determined spectrophotometrically using a Beckman DBG spectrophotometer and an extinction coefficient at 260 nm of 7.66 mg^{-1} ml cm^{-1} (Gesteland and Boedtker, 1964). Therefore one absorbancy unit (A_{260} unit) contained 2.18 x 10^{13} particles. Usually a purified R17 phage preparation consisted of 0.5 ml of solution containing 5 - 10 x 10^{14} particles/ml.

^{32}P-labelled R17 was prepared using low phosphate CTMM as the culture medium. At 5 min post-infection, a neutralized solution of ^{32}P inorganic phosphate (10 mCi) was added and the incubation and purification procedure was as previously described. Freshly purified phage R17 preparations had a specific activity of 2 x 10^{-6} cpm/particle.

R17 phage labelled with ^3H-guanosine in the RNA or ^3H-amino acids

in the coat and A protein were similarly prepared using CTMM+aa or CTMM-aa as the culture medium respectively. At 20 min post-infection (10 min post-infection), 0.5 mCi of ^{3}H-guanosine (or 5 mCi ^{3}H-L-amino acid mixture) was added and the phage was purified in the usual fashion. The specific activity of such ^{3}H-labelled phage preparations was similar to that obtained for freshly purified ^{32}P-labelled phage. A detailed procedure for the purification of cold or radioactive labelled R17 phage has been reported by Krahn et al. (1972), Wong (1975), and Wong and Paranchych (1976).

D. RADIOISOTOPE COUNTING

Dry samples such as air-dried filter discs from attachment assays were counted for 5.0 min in 5.0 ml toluene-based scintillation fluid (prepared by adding 4.0 g Omnifluor [New England Nuclear] to 1.0 litre of scintillation grade toluene) in a Beckman LS-250 liquid scintillation spectrometer.

Aqueous samples were assayed by combining 0.5 ml of sample with 5.0 ml Bray's scintillation fluid (180 g napthalene, 12 g PPO (2,5-diphenyloxazole), 0.6 g POPOP (p-Bis(2-(5-phenyloxazolyl)-benzene)), 300 ml methanol per litre of 1,4-dioxane [Fisher-Scientific]). The efficiency of counting was >95%, 85% and 30% for ^{32}P, ^{35}S and ^{3}H isotopes respectively.

Single isotope restricted channels were used for double-labelling experiments and counts were corrected for overlap.

Similarly prepared samples were monitored for efficiency of counting using an external standard.

All samples were corrected for background radioactivity by using

controls identical to the samples but containing no radioactivity.

E. PHAGE SPOT TEST

The sensitivity of a particular bacterial strain to bacteriophage R17 was tested by spreading 0.5 ml of a bacterial culture in exponential phase on a TSB hard agar plate with a sterile glass spreader. A drop of clarified crude phage lysate was placed on the plate and it was incubated at 37° overnight. Plaque formation was indicative of phage sensitivity.

F. R17 PHAGE ATTACHMENT ASSAYS

1. Filtration assay for cell-associated and cell-free F pili

The filtration assay employed to measure phage-pili complex formation was essentially that described by Danziger and Paranchych (1970a). Unless otherwise specified 0.5, 1.0 and 2.0 ml samples of cultures (4 - 8 x 10^8 cells/ml) were diluted to 2.0 ml with TMM. Each sample received 0.1 ml of 0.1 M $MgCl_2$ and 0.1 ml of ^{32}P- or ^{3}H-labelled R17 (containing 10^{12} particles of R17 and 4 - 8 x 10^5 cpm, depending on the age of the radioactive phage preparation). The samples were incubated at 4°C for 40 min to measure phage adsorption to pili. During this time, Gelman GA-6 triacetate filters (0.45 μ) were placed in a series of 20 ml filter head units (Millipore Co.) and were washed with 5 ml of water, 5 ml of 5 mg % (w/v) bovine serum albumin to reduce non-specific binding of free phage particles to the filters, and 5 ml of TMM + Mg^{++}. The sample was passed through the filter under a vacuum of 6 in of mercury (Gast pump) and the sample tube was

rinsed with 5 ml of TMM + Mg^{++}. The filter was washed with two additional portions of TMM + Mg^{++} and the filters were thoroughly drained of liquid. The filters were mounted on stainless steel pins until thoroughly dry and were counted in 5 ml of Omnifluor scintillation fluid. Two samples containing only phage were filtered, dried and counted to measure background levels of radioactivity. Also, duplicate 0.1 ml samples of phage solution were dried on Whatman 3 mM filter discs and counted to give the amount of input radioactivity. The slope of a plot of sample volume (0.5, 1.0 and 2.0 ml) versus radioactivity (cpm/sample) gave the level of attachment for a 1.0 ml sample of cells corrected for nonsaturation of the available F pili with phage at high cell densities.

Alternatively, samples containing cell-free F pili were assayed for radioactive R17 phage attachment using the following modifications. Sample volumes were reduced to 1.0 ml to which 50 µl of 0.1 $MgCl_2$ and 50 µl of radioactive-labelled R17 (5×10^{11} particles/sample) were added. The mixture was incubated at room temperature for 40 min and was passed through the filter accompanied by only one 5 ml portion of TMM + Mg^{++} to rinse the sample tube and wash unattached R17 phage from the filter. The filters were dried and counted as previously described.

2. Centrifugation assay for cell-associated F pili

In this assay, cell-associated pili are saturated with ^{32}P-R17 particles (10^4 particles/cell) and separated from cell-free pili by centrifugation. This allows for a direct comparison between samples of the same or differing strains of cells for available F pili on the

cell surface (Paranchych, 1975).

A 1 ml portion of cells (0.4 A_{650} units) was diluted with 1 ml of cold TSB broth and placed in an icebath. Each sample received 20 µl of 0.5 M $MgSO_4$ and 0.1 ml of ^{32}P-R17 (2×10^{12} particles) and was allowed to incubate at 5° for 40 min. A 0.1 ml portion from each sample was spotted on a Whatman 3 MM filter disc to determine the input of radioactivity and the rest of the sample was centrifuged at 10,000 x g for ten minutes. The supernatant was decanted and drained and the sides of the centrifuge tubes were blotted dry. The pellets were resuspended in 0.2 ml TMM + Mg^{++} and 0.1 ml was spotted and dried on a filter disc. The filters were dried and counted in 5.0 ml toluene scintillation fluid. ED2601 (F^-) was used to determine background attachment and the level of attachment of ED2602 (JCFLO) was taken as 100%.

G. ASSAY FOR PLASMID TRANSFER

Log-phase cultures (2×10^8 cells/ml) of donor and recipient cells were mixed at a ratio of 0.1 ml donor cells/0.9 ml recipient cells and incubated at 37° for 30 min with gentle shaking. The mating mixture was then chilled to 4° and plated on various selective minimal medium plates at appropriate dilutions. After incubation at 37° for 24 - 48 hr, the number of conjugants/plate was enumerated and reported as the number of conjugants per 100 donors of original mating culture.

H. CENTRIFUGATION EQUIPMENT

All low speed centrifugation procedures (<15,000 rpm) were performed using a Sorvall RC2-B centrifuge with a thermostatic control set at 5°,

using an SS-34 or GSA rotor depending on sample volume. Samples were spun in 50 ml polypropylene tubes or 15 or 30 ml Corex glass tubes with rubber adapters (Sorvall Inc.) or in 250 ml Nalgene bottles with screw cap lids.

High speed centrifugation procedures were done using a Beckman L2 65-B ultracentrifuge set at 5°. All gradients were formed using swinging bucket rotors SW50.1 (6 x 5 ml buckets), SW27.1 (6 x 18 ml buckets) and SW27 (6 x 30 ml buckets) with Beckman cellulose nitrate tubes of the appropriate size. Procedures requiring pelletting of material used a fixed angle 60 Ti or S30 rotors equipped with screw top polypropylene tubes of an appropriate size.

I. CsCl DENSITY GRADIENT CENTRIFUGATION

Routinely, the sample was dissolved in 4.5 ml of SSC buffer pH 7.0 in a Beckman cellulose nitrate tube to which a pre-weighed amount of dry, ultrapure CsCl (Gallard, Schlesinger) was added to give the desired density at 20° as determined by the index of refraction at 20°. The total volume was 4.7 - 4.9 ml. The gradient was formed in an SW50.1 rotor at 5° at 35,000 rpm for 22 hours.

The tube was punctured at the bottom with a gradient dripper (Buchler Instruments) and 15 drop fractions were collected (0.2 ml in each) in 13 mm test tubes.

The index of refraction at 25° was determined for even numbered fractions using a 10 µl sample and a Bausch and Lomb refractometer connected to a Haake thermostatically-controlled circulating water bath. This value ($n_D^{25°}$) was converted to density at 5° ($\rho^{5°}$) using the equations $\rho^{5°} = 1.027(\rho^{20°}) - 0.0268$ (Beard, personal communication) and

$\rho^{20°} = 10.2402\ n_D^{25°} - 12.6483$ (International Critical Tables). A table converting $n_D^{25°}$ to $\rho^{5°}$ appears in Appendix A. The contribution of SSC buffer to the index of refraction was negligible.

J. SUCROSE GRADIENT TECHNIQUES

The buoyant density of pili in sucrose was determined using a discontinuous sucrose gradient containing layers of 3.0 ml of 70, 60, 50, 40 and 30% (w/v) sucrose in SSC, pH 7.0. The sample of pilus solution (1.5 ml containing 5 - 6 mg of pili) was layered on the gradient and the gradient was centrifuged for 72 h at 22,000 rpm at 5° using an SW27.1 rotor. The gradient tube was subsequently punctured at the bottom and the contents of the tube were pumped out into 0.5 ml fractions using an LKB Perpex peristaltic pump. The refractive index of each fraction was read at 25° and this value was converted to density in sucrose at 5° by comparing the $n_D^{25°}$ to a standard curve relating density of various concentrations of sucrose at 5° (CRC Handbook of Biochemistry, 1968) to the index of refraction of solutions made at 5° and subsequently warmed to 25°. A table comparing $n_D^{25°}$ to $\rho^{5°}$ for sucrose is given in Appendix B.

Sedimentation velocity sucrose gradients were performed by layering 0.2 ml of sample on top of a 4.5 ml continuous 5 - 20% (w/v) sucrose gradient (in SSC buffer, pH 7.0) formed with a split chamber gradient maker (Buchler Instruments) in a 5.0 ml cellulose nitrate tube. The gradient was centrifuged in an SW50.1 rotor at 45,000 rpm at 5° for 45 min and was dripped in the usual manner.

K. ELECTRON MICROSCOPY

Specimens for electron microscopy were prepared by placing a drop of solution on a 200 mesh copper grid held by fine-tipped forceps for 20 to 60 sec and draining the drop away with filter paper. The grids had previously been coated with a 2% parlodion film (in amyl acetate), thoroughly dried, coated with carbon and glowed using a Balzers Micro BA3 carbon evaporation unit to promote sample adhesion to the grid. The specimen was negatively stained with 2% sodium phosphotungstate (pH 7.0) for 10 to 20 seconds and was drained with filter paper. The specimens were examined with a Philips EM300 transmission electron microscope at 4 µA, 80 kV. The longer times for sample application refer to samples containing cells while the longer staining times were used for cell-free purified pili specimens.

L. ISOELECTRIC FOCUSING

Isoelectric focusing was conducted according to the method of Vesterberg and Svensson (1966) as modified by Beard et al. (1972). The pilus preparation (2 - 3 mg) was dialyzed for 3 days against 6 X 2 litres of 1% glycine (w/v) in distilled deionized water. The dialyzed pilus solution was incorporated into a 50 ml solution of 50% sucrose (w/v) containing 4% (w/v) ampholyte (LKB ampholine, pH range 3.0 to 10). A gradient was formed in an LKB 8101 100 ml column with the anode at the bottom of the column. The anode solution consisted of 0.2 ml concentrated H_2SO_4 plus 12 g sucrose in 14 ml of water. An LKB gradient maker with the 50% sucrose solution described above in one chamber and 50 ml of water in the other chamber was used to form the gradient. The cathode solution (0.2 ml triethanolamine in 10 ml water) was layered on

top of the gradient. The column was focused at 350 volts (10 - 20 ma) for 24 hours using an LKB 3371 D power supply at which time the current had fallen to 2 - 3 ma and a band near the bottom of the column was visible. The band was sharpened by increasing the voltage to 450 volts for 5 hours. The procedure was carried out at 10° using a thermostatically controlled Haake circulating water bath and a KR30 refrigeration unit (PolyScience Corporation). At the end of the run, the anode was closed off and the column was pumped out into 1.0 ml fractions using an LKB peristaltic pump. The pH of each fraction was recorded with a Radiometer 26 pH meter equipped with a glass microelectrode and the absorbance at 280 nm was determined on every second fraction using a Beckman DBG spectrophotometer.

The polarity of the column was reversed by reversing the anode and cathode and using 10 ml of 1% (v/v) sulfuric acid as the anode solution and 0.4 ml triethanolamine in 14 ml of water containing 12 g of sucrose.

M. PAPER CHROMATOGRAPHY

A piece of Whatman 1 MM paper (23 by 56 cm) with the origin 10 cm from one end was spotted at 3 cm intervals with hydrolyzed pilin (0.5 - 2.0 mg) and 20 µg of the following standards: glucose, fructose, galactose, glucosamine, galactosamine, N-acetylglucosamine, ribose, arabinose, fucose, rhamnose, xylose, sucrose, lactose, 3,6-dideoxyglucose, 3-deoxyglucose (the unusual sugars being the gift of Dr. R.U. Lemieux). The required amount of material was applied to the paper in successive 2 µl amounts with the paper being dried between applications. The prepared chromatogram was pre-equilibrated in a saturated chromatography

tank for four hours at which time solvent was introduced and the chromatogram was run (by descending chromatography) for 16 hours at room temperature. The solvent was ethyl acetate:pyridine:water (12:5:4).

The chromatogram was dried and sprayed with one of the following sprays:

(a) Anisaldehyde-sulfuric acid (Stahl and Kaltenbach, 1961). A solution containing 90 ml ethanol (95%), 15 ml anisaldehyde (Eastman Organic Chemical Co.), 5 ml concentrated sulfuric acid and 1.0 ml glacial acetic acid. The chromatogram is heated at 100° for 5 - 10 min to produce spots of various colours, characteristic for a wide range of sugars. The spray can detect 1 µg of sugar.

(b) $AgNO_3$-NaOH (Trevelyan et al., 1950). The chromatogram is sprayed with 1 ml of a solution of saturated $AgNO_3$ dissolved in 199 ml of acetone by the addition of 5 ml of water. The chromatogram is dried and sprayed with 2% NaOH in methanol with a small amount of water to affect solution. The chromatogram is heated at 100° for several minutes to produce black spots on a buff background. The sensitivity for reducing sugars was 1 µg, less for non-reducing sugars.

N. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was performed at room temperature by the method of Weber and Osborn (1969). A solution (25 ml in volume) consisting of 12.5% acrylamide and 0.34% N,N'-methylenebisacrylamide in 0.05 M sodium phosphate, pH 7.2, containing 0.1% SDS was filtered through a 0.2 µ Nalgene disposable filter unit. The

polymerization reaction was catalyzed by the addition of 0.07% ammonium persulfate and 0.02% N,N,N',N'-tetramethylethylenediamine which occurred in approximately fifteen minutes. The gel solution was syringed into 12 cm glass tubes (inner diameter 5 mm), and capped on the bottom with Parafilm, to a height of 10 cm. A layer of water was introduced over the gel solution with a small syringe with a bent needle to achieve a smooth gel surface for application of the sample. After the gels had hardened, the Parafilm was removed and the gels were stored in the cold in a beaker of buffer solution.

A total of 10 - 50 µg of protein was dissolved in 50 µl of 0.2% SDS and 0.02% dithiothreitol in a glass vial and was heated in a boiling water bath for 5 min. The vials were cooled and 50 µl of 60% glycerol (v/v) containing 0.005% bromphenol blue was added to the samples. The samples were applied to the top of the gels which previously had been drained of water and inserted symmetrically in a circular disc gel electrophoresis unit (12 sample maximum [Hoefer Scientific Instruments]). Buffer (0.05 M sodium phosphate, pH 7.2, 0.1% SDS) was layered over the sample and the bottom and top tanks were filled with buffer. The bottom tank was equipped with a jacket through which tap water was passed. The gels were run at 5 mA/tube until the dye front was 1 cm from the bottom of the tube (3 hours). The gels were removed from the tubes using a stream of water and a syringe fitted with a cannula and were stained using one of the following stains.

O. STAINS FOR POLYACRYLAMIDE GELS

1. Coomassie brilliant blue for protein

A modified version of the stain described by Fairbanks et al. (1971) using one tenth of the normal concentration of Coomassie brilliant

blue was used. The gels were soaked in 50 ml of 0.005% (w/v) Coomassie brilliant blue in 10% isopropanol, 10% acetic acid for 24 hours. The gels were then stained in 0.005% Coomassie brilliant blue in 25% isopropanol, 10% acetic acid for another 24 hours. This method gave visible protein bands on a relatively faint background which could be removed in a few hours by destaining in 10% acetic acid.

Rapid staining and destaining was accomplished by staining the gels at 37° for 1.5 hours in 0.25% Coomassie brilliant blue in 50% methanol, 10% acetic acid and using a rapid gel destainer (Canalco) for 30 min at room temperature using 5% methanol, 7.5% acetic acid.

2. Methyl green stain for phosphoprotein

The method of Cutting and Roth (1973) was used to detect the presence or absence of phosphoprotein in polyacrylamide gels. The gels were rinsed in 10% sulfosalicylic acid (SSA) for 24 hours to remove contaminating phosphate ions and then soaked in 0.5 M $CaCl_2$, 10% SSA for two hours and washed three times in distilled water. The gels were incubated at 60° in 0.5 N NaOH for 30 min, washed twice at 10 min intervals with 1% ammonium molybdate, soaked in 1% ammonium molybdate, 1 $NaHCO_3$ for 30 min and stained in 0.5% methyl green in 7% acetic acid for 30 min. The gels were destained and stored in 10% acetic acid. α-Casein was run as a phosphoprotein control to test the sensitivity of the procedure which could detect 1 nmol of phosphate or 1 phosphate residue in 300 amino acid residues. Nonphosphoproteins (bovine serum albumin) appeared as white bands on a faint green background while phosphoproteins (α-casein) appeared as dark green bands.

3. Periodic acid-Schiff stain for glycoprotein

The periodic acid-Schiff reaction described by Clark (1964)

amplified by the Ortec Application Note AN32 (1972) consisted of rinsing the gels in 10% acetic acid overnight followed by 0.2% periodic acid for 45 min at 5°. The gels were placed in Schiff reagent for 2 hours at 5° and destained and stored in 10% acetic acid. The Schiff reagent was reduced and acidified basic fuchsin. A 0.5% solution of basic fuchsin (200 ml) was dissolved in a boiling water bath for 5 min. After the solution was cooled, filtered and 20 ml of 1 N HCl had been added, it was cooled to room temperature, 1 g sodium metabisulfite was added, and it was placed in the dark for 18 hours. Activated charcoal (2 g) was added, and the solution was shaken and filtered to give a clear solution. The stain produced red bands on a clear background as seen when rabbit serum was used as a source of glycoprotein. The test is highly sensitive for aldehydes.

4. Oil-red O stain for lipoproteins

Gels were soaked in 60% ethanol saturated with oil-red O for 24 hours and were destained in 50% ethanol followed by 10% acetic acid (Abodeely et al., 1971). Two µl of rabbit serum when run on a gel gave red bands of very high molecular weight (Abodeely et al., 1971).

P. ANTISERA PREPARATION

To ensure that absolutely pure pili were used in the preparation of anti-pilin antiserum, purified PAK and PAK/2PfS pili were subjected to a final purification step of SDS-polyacrylamide gel electrophoresis. Preparative gel electrophoresis was similar to the procedure for SDS-polyacrylamide gels described above except for the following differences. The gels were 1 cm in diameter and 6 cm in length and could accommodate a 0.5 ml sample containing 300 µg of SDS-treated pili.

The buffer was 0.05 M sodium phosphate, pH 7.2, 0.1% SDS. In each sample of SDS-treated pili was 20 - 30 µg of prestained pilin which had the same mobility as unstained pilin. The prestained pilin was prepared according to the method of Griffith (1972). To 1 ml of pilus solution (2 - 5 mg), 1.0 ml of 1 M disodium phosphate, pH 9.2, was added as well as 0.5 ml of 10% Remazol brilliant blue in 10% SDS. The mixture was boiled for 15 min, cooled and the stained protein was precipitated by the addition of 5 ml of 0.1 N HCl in spectrograde acetone. The protein was harvested by low speed centrifugation and redissolved in 2 ml of 0.1% SDS.

After the pilin band had migrated approximately 3 cm down the gel (10 ma/tube for four hours) the run was terminated and the pilin band was excised from the gel. The pilin bands from four gels (1.2 mg total) were suspended in 3 ml of gel buffer and pulverized by forcing the slices repeatedly through an 18-gauge needle. The gel was mixed thoroughly with 3 ml of Freund's complete adjuvant and 2.0 ml of this homogenized preparation was injected subcutaneously into the dorsal area of three New Zealand white rabbits. The injections were repeated three more times at 6 week intervals and at five months the rabbits were bled from the ear using gentle suction. The blood was allowed to stand at 5° for several hours until it had formed a firm clot and was gently removed by a wooden sterile stick inserted in the clot. The resultant serum was stored at -20°.

Q. MICROIMMUNODIFFUSION ANALYSIS

Double gel diffusion analysis was carried out on glass plates (5 X 5 cm) on which rested a plastic template consisting of 6 wells

encircling a center well, each 10 µl capacity, supported by an 8-pound test monofilament fishing line. A layer of hot 1% agarose (Induboise A45, Industrie Biologique Française) in 0.1 M sodium phosphate, pH 7.2 and 0.15 M NaCl was introduced between the glass plate and the template. After the agarose had cooled, the wells were sucked clean of excess agarose with a Pasteur pipet. The microdiffusion plates were stored in a water-saturated atmosphere at 5°. The serum was applied undiluted to the center well and the antigens were placed in the surrounding wells. The plates were incubated for 4 days at room temperature in a water-saturated atmosphere.

The templates were removed from the surface of the agarose and the agarose was rinsed for 48 hours in saline at 5° and 24 hours in distilled water (5°). The plates were stained with 0.005% Coomassie brilliant blue in 10% isopropanol, 10% acetic acid at 5° for one hour or until blue bands were visible. Destaining was in 10% acetic acid at 5°, after which the agarose was allowed to dry to the surface of the glass.

R. ACID AND BASE HYDROLYSIS

Routine acid hydrolysis was performed on 1 mg amounts of salt-free lyophilized pili. The samples were hydrolyzed in 6 N HCl containing 0.1% phenol for 22 hours at 110° in evacuated, sealed glass tubes. After hydrolysis, the tubes were opened and lyophilized over NaOH pellets.

Alkaline hydrolysis was by the method of Hirs (1967). Pili samples (1 mg of salt-free lyophilized material) were placed in Pyrex tubes containing 0.15 ml of 1.35 N NaOH and autoclaved for 20 min at 15 lb/in^2. The samples were then cooled and acidified with 0.25 ml of glacial acetic acid.

CHAPTER III

PURIFICATION OF F PILI FROM CULTURES OF *E. COLI*

A. INTRODUCTION

The following chapter describes the author's attempts at purifying F pili. Earlier work by Brinton and his coworkers (Brinton, 1965, 1971; Brinton and Beer, 1967; Novotny *et al.*, 1969a) suggested that F pili were continually being synthesized and released by the cell resulting in an accumulation of F pilus material in the supernatant. However the lack of a quantitative assay for F pili either in a bacterial culture or in a concentrated cell-free solution made estimation of the available F pili in a culture difficult.

B. RESULTS AND DISCUSSION

1. Choice of bacterial strains

The purification of F pili is greatly simplified if the host strain is derepressed, and, therefore, expresses its full capacity for F pilus synthesis. Also the host strain should produce no other extracellular appendages such as type 1 pili or flagella.

The first attempts at purifying F pili used HB11 (Brinton and Beer, 1967; Valentine *et al.*, 1969), a B/r *lac*$^-$ strain of *E. coli* carrying the wild type F*lac* plasmid, HBFLO, which produced neither type 1 pili nor flagella (*fim*$^-$, *fla*$^-$), a definite advantage since these are the main contaminants of F pili preparations. However HB11 was poorly characterized genetically in comparison to ED2602, a K12 strain carrying the F plasmid JCFLO which had been used by Willetts and his coworkers in elucidating the F transfer system (Achtman *et al.*, 1971).

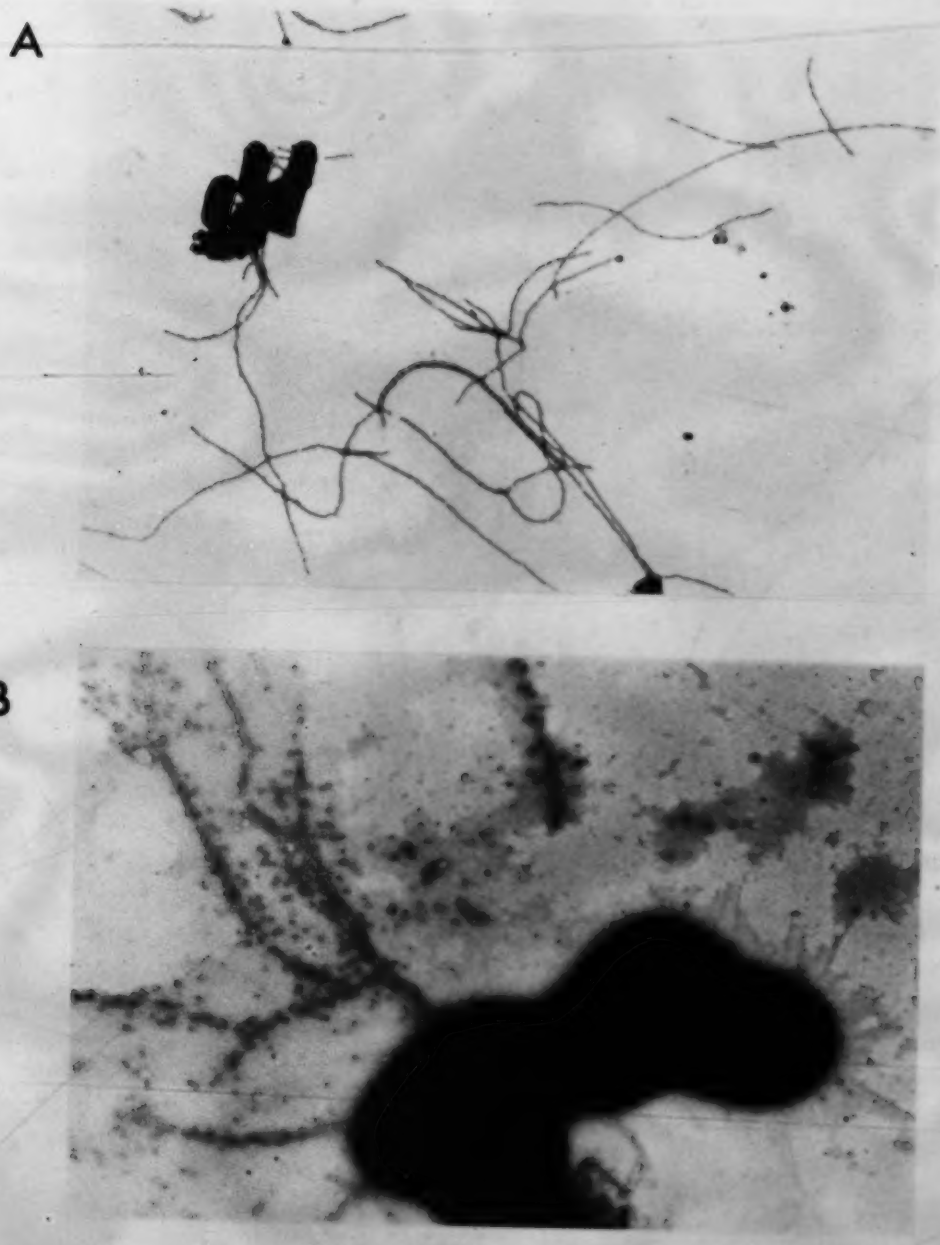


FIGURE 2. Electron micrographs of cultures of HB11 and ED2602 cells.

Cultures of HB11 (A) and ED2602 (B) were grown in broth to 2 x 10^8 cells/ml and labelled with R17 particles (2000 p/cell). The micrograph of HB11 is taken at low magnification to illustrate the large amount of free F pili in the culture. The micrograph of ED2602 contains a cell producing type 1 pili as well as a small fragment of flagella. Magnification: A, X2800; B, X17,500.

Furthermore, it was a poor donor when assayed for transfer ability and gave cloudy, indistinct plaques when lysed with R17 bacteriophage.

Therefore, HB11 was cured of its F plasmid using acridine orange as outlined by Miller (1972). HB11 F^- colonies were isolated and tested for their inability to grow on a lactose-based minimal medium, their insensitivity to the F-specific phages R17 and M13 and their lack of F pili as assayed by ^{32}P-R17 attachment assay and by electron microscopy.

The strain ED2687 was the transconjugant isolated from a cross between E. coli ED2602 X HB11F^-. It was isolated on the basis of its ability to grow on a lactose-based minimal medium containing streptomycin but no histidine, tryptophan or lysine, as well as the presence of F pili as measured by R17 phage sensitivity and electron microscopy.

The principal strains used for the production of F pili were HB11, ED2687 and ED2602, the first two strains sharing a common host strain, HB11F^-, while the last two have the plasmid JCFLO in common. An electron micrograph of strains HB11 and ED2602 is given in Figure 2.

The amount of F pili produced by these three strains was measured by electron microscopy, ^{32}P-R17 phage attachment using the centrifugation assay for cell-associated F pili only, and by the efficiencies with which they plate R17 and transfer their plasmid to a recipient strain relative to ED2602 (Table 3). It should be noted that while ED2602 had the most cell-associated F pili compared to HB11 and ED2687, it also produced type 1 pili and small amounts of flagella while ED2687 and HB11 did not do so. Also HB11 cultures characteristically contained large amounts of free F pili in the background of specimens of cells prepared for electron microscopy.

TABLE 3

A Comparison of the Level of Piliation on the Cell Surface of Six Bacterial Strains

Strain, Plasmid	Efficiency of Plating of R17 (%)	Efficiency of Transfer (%)	^{32}P-R17 particles attached/ cell	F pili/ cell
ED2601	0	0	0	0
ED2602 JCFLO	100	100	625	0.80
ED2687 JCFLO	69	1.7	508	0.75
ED2692 JCFL*traD*$_8$	0	0	945	1.70
HB11 HBFLO	96	1.3	529	0.76
WP156 Hfr	100	n.d.*	449	n.d.*

*not determined

Each culture was grown in TSB broth to 2 x 10^8 cells/ml and chilled. A sample of each culture was assayed for number of cells/ml (colony count); transfer ability using ED2601 as the recipient cell; ^{32}P-R17 particles attached/cell using the centrifugation assay; and efficiency of plating R17 using the cells as a bacterial lawn. The efficiency of plating and transfer ability were expressed as percent where ED2602 was taken as 100%. The number of F pili/cell was estimated by counting the F pili attached to 50 cells visualized by electron microscopy. The details for these methods are given in Chapter II.

The strain ED2692 is included in Table 2 since it produces nearly twice the amount of F pili as ED2602 as seen by electron microscopy. This is due to a traD mutation which renders ED2692 transfer-deficient and resistant to R17 although still capable of attaching phage particles. ED2692 is a close relative of JC6449, the strain used by Brinton in his chemical analyses of F pili (Brinton, 1971). Unfortunately, ED2692 was not available until the latter stages of this work and was used very little in preparing F pili concentrates. The Hfr strain, WP156, used in this laboratory for propagating phage stocks, is included because it is a more efficient host for R17 phage. A summary of the strains used in this work along with genotypes is given in Table 2.

2. Growth conditions for optimal F pilus production

Production of F-type pili usually parallels the growth of the host cells in a culture (Ippen and Valentine, 1965). Pili synthesis increases most rapidly during the exponential phase of cell growth and reaches a maximum or plateau during late exponential phase or early stationary phase of growth (Ippen and Valentine, 1965; Brinton and Beer, 1967). A general phenomenon of pili outgrowth is the stability of released F pili in the medium for many hours, while the level of piliation on the cells, as measured by electron microscopy or RNA-phage adsorbing capability (Brinton and Beer, 1967; Valentine et al., 1969; Beard et al., 1972) reaches a maximum at 5 - 7 x 10^8 cells/ml and drops drastically during early stationary phase (2 x 10^9 cells/ml) to 4% of the maximum level of cell-associated piliation (Donelian, 1972). This decrease in cell-associated F pili at high bacterial cell density coincides with the production of F^- phenocopies (Jacob and Wollman, 1961; Hayes, 1964) which are poor donors (Novotny et al., 1969b; Ou and Anderson, 1970)

and are relatively insensitive to RNA phage infection (Krahn and Paranchych, 1971).

Brinton (1965; Brinton and Beer, 1967; Brinton et al., 1964; Novotny et al., 1969a) postulated, on the basis of the reappearance kinetics of F pili after mechanical shearing, that F pili at early exponential phase of the culture grow out and drop off the cell every 4 or 5 minutes. The fact that F pili show a narrow range in length variation (1.2 - 1.3 microns), unlike type 1 pili or flagella, whose length increases throughout the generation time of the cell, supported this claim of continual pili renewal. Valentine et al. (1969) reported that 50% of the total F pili in a broth culture of HB45 bacteria was free F pili at 5×10^8 cells/ml. Donelian (1972) reported a lower value of 30% for HB11 F pili grown in a synthetic medium to 5×10^8 cells/ml. Donelian also showed that the accumulation of free F pili in a bacterial culture was not due to mechanical removal of the pili by agitation of the culture used to achieve good levels of aeration.

Levels of piliation are affected by temperature, the optimum occurring between 37° and 42°, whereas no pili are synthesized by cells grown at room temperature (Novotny and Lavin, 1971). Also a rich medium supports higher levels of piliation than a synthetic medium (Tomoeda et al., 1975) and cultures grown under limited aeration are better producers of F pili than aerated cultures (Curtiss et al., 1969; Tomoeda et al., 1975). This last statement opposes the collective findings of Brinton, Beard, Donelian, and Novotny and coworkers, who routinely used well-aerated cultures to produce maximum amounts of F pili. Novotny et al. (1969b) have suggested that F pilus outgrowth is regulated by aerobic metabolic processes which are slowed down during stationary

phase growth due to competition for existing nutrients and oxygen.

These previous studies were used as the starting point for developing a purification procedure for F pili. Donelian (1972) studied extensively the growth characteristics and harvesting of F pili produced by HBl1 and his findings were adopted by this author. However a few modifications, presented below, were made.

Preliminary studies showed that the components of broth badly contaminated F pili preparations with carbohydrate and protein material. This was done by simply adjusting the pH of sterile broth to 4.0 and allowing it to stand overnight in the cold as described in the acid precipitation of F pili from broth supernatants by Donelian (1972). A large precipitate composed of carbohydrate and protein as indicated by the orcinol (Ashwell, 1957) and Lowry (Lowry et al., 1951) colorimetric assays was obtained.

Thus, a synthetic medium was sought which supported a level of F pilus synthesis similar to that achieved with broth cultures. The conclusion drawn from this work showed that on the average, synthetic media achieved only 75 - 80% of the amount of F pili produced by cultures grown in trypticase soy broth (TSB), providing a complete amino acid supplement such as 1% Earle's amino acid mixture (Difco) or 1% hydrolyzed casein (Casamino acids [NBCo]) was added to the medium. The sugar used as a carbon source could be either glucose or lactose, both supporting equivalent levels of piliation. There appeared to be no special requirement for various salts or vitamins other than those supplied by the ingredients in CTMM. The composition of broth-based and synthetic media as well as the conditions for the storage and propagation of bacterial strains has been given in Materials and Methods.

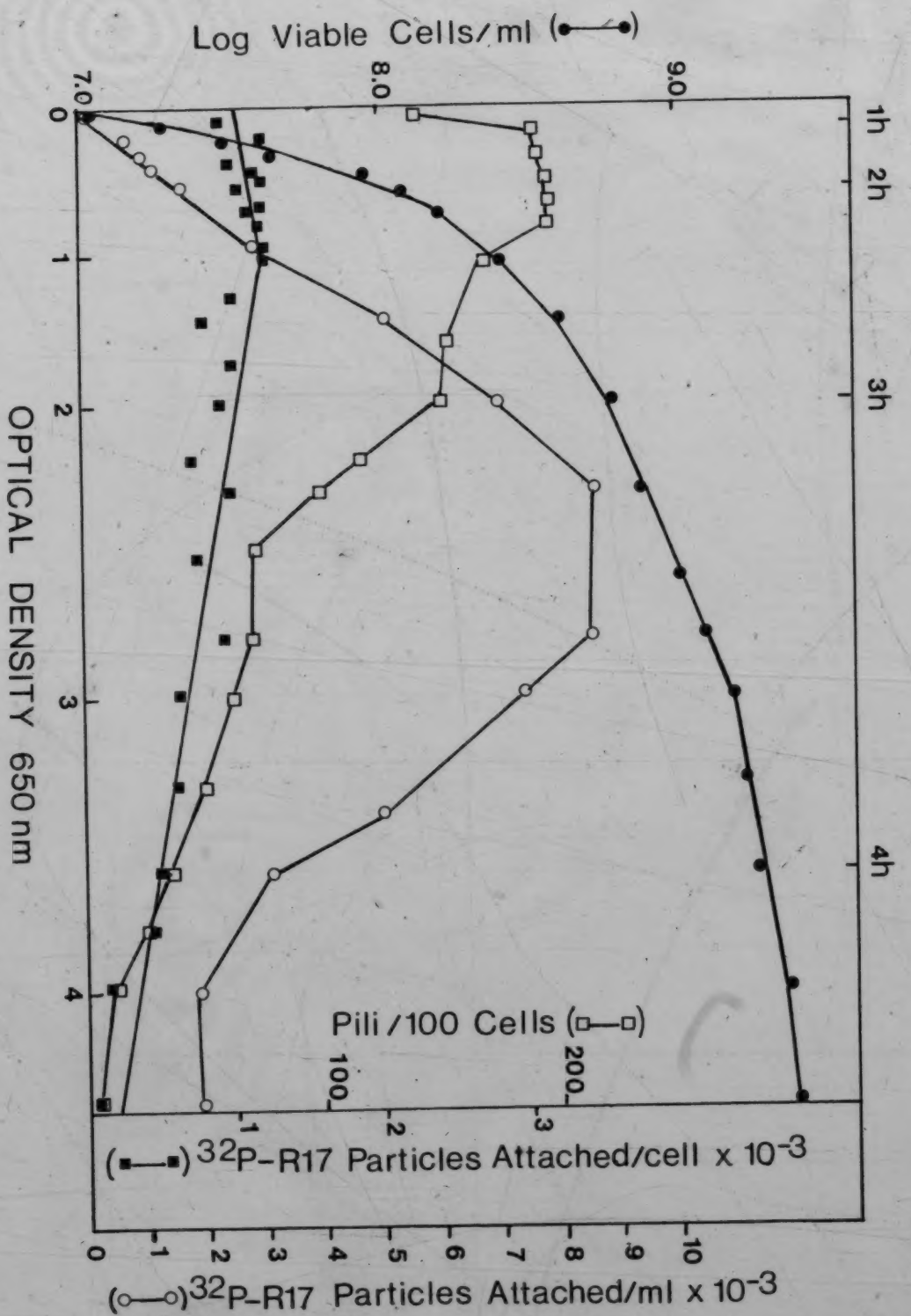
The relationship between cell density and the level of piliation in terms of RNA phage adsorption capacity has been previously explored (Brinton and Beer, 1967; Valentine et al., 1969; Beard et al., 1972; Donelian, 1972). The optimal level of F pilus synthesis occurs during early log phase, and then, depending on the strain being studied, piliation decreases at varying rates as cell growth enters the stationary phase. This was also shown to be the case for the F plasmid JCFLO in ED2687. As seen in Figure 3, maximal piliation, as determined by measuring cell-associated pili, occurs at cell densities of 2×10^8 cells/ml or less. Thereafter, the level of cell-associated pili begins to decrease. However, the maximal amount of F pili/ml of culture, which includes cell-free pili, occurs during early stationary phase, i.e., $1.0 - 1.6 \times 10^9$ cells/ml, and then decreases dramatically in a manner similar to the strains previously studied.

3. Removal of F pili from the cells

While 30 - 50% of the F pili in a culture are free from the cells (Valentine et al., 1969), the percentage of F pili recovered can be raised to 65 - 70% by shearing the attached F pili off the cells. Brinton (1965) and Valentine and Strand (1965) first suggested high speed blending to shear off the F pili and the conditions for the most efficient use of a blender such as a Sorvall Omnimixer were studied by Novotny et al. (1969a). They proposed that 40 ml samples of F^+ cells required 2 minutes blending at 2200 rpm for efficient removal of F pili without excessive shearing of the F pili into small fragments or undue contamination with other surface structures such as flagella or type 1 pili. Donelian (1972) modified this procedure for large scale cultures

FIGURE 3. The relationship between levels of piliation and cell density in E. coli ED2687.

A log-phase culture of ED2687 cells was diluted in TSB to a density of 1×10^7 cells/ml, then grown at 37° in a rotary water bath. At fifteen minute intervals, samples were taken and assayed for optical density at 650 nm and the number of viable cells/ml to give the growth curve (●-●). In addition, a sample of cells was diluted when necessary with cold medium to 5×10^7 cells/ml in a total volume of 2.0 ml. ^{32}P-labelled R17 was added to each sample at a ratio of 10^4 particles/cell and assayed for the number of R17 particles attached/cell (■-■) using the centrifugation assay. Similarly a duplicate sample was assayed for to total ^{32}P-R17 attachment/ml of culture (o-o) using the filtration assay and ratio of 10^3 R17 particles/cell. Aliquots of each sample were also treated with formaldehyde, then examined by electron microscopy (as described in Materials and Methods) to determine the number of cell-associated F pili per 100 cells (□-□).


Log Viable Cells/ml (●—●)
7.0
8.0
9.0
1h
2h
3h
4h
OPTICAL DENSITY 650 nm
0
1
2
3
4
Pili/100 Cells (□—□)
100
200
1
2
3
(■—■) 32P-R17 Particles Attached/cell x 10-3
0
1
2
3
4
5
6
7
8
9
10
(○—○) 32P-R17 Particles Attached/ml x 10-3

by using a continuous flow blender cup. This device was much more convenient than blending the cells in small batches but the shearing speed and flow rate which he recommended were very slow, i.e., four hours for 15 litres of culture.

Thus conditions were sought which maximized pilus removal from the cells without shearing the pili into shorter fragments. The optimum conditions were a flow rate of 200 ml/min and a blender speed of 8200 rpm which resulted in an 83% loss in R17 phage attachment capability by the blended cells with no visible destruction of pili as visualized by electron microscopy. The propensity of F pili to nonspecifically bind to the cells was overcome by chilling the culture and blending cup as suggested by Novotny et al. (1969a). Also, the addition of 10 mM urea to the chilled culture improved the yield of F pili in the supernatant by 13%. This effect was supposedly due to the ability of the urea to dissociate F pili from cells and cellular debris, i.e., solubilize the pili and therefore increase the amount of F pili in the supernatant.

This procedure was used to prepare cell-free supernatants from which the F pili were concentrated by precipitation or two-phase partitioning techniques.

4. Concentration and purification of F pili from the supernatant

Several distinct procedures for concentrating F pili from a cell-free supernatant have previously been reported. The first widely used procedure involved precipitating F pili at their isoelectric point, pH 4.0, from chilled broth supernatants (Brinton and Beer, 1967; Valentine et al., 1969; Donelian, 1972), followed by CsCl isopycnic centrifugation. These authors concluded that the resulting F pili concentrate

was grossly contaminated with carbohydrate and protein material and that several cycles of CsCl gradient centrifugation caused severe losses of F pili material (Valentine et al., 1969).

In the present study, an effort was made to improve the foregoing purification procedures by seeking a more effective means of concentrating pili from crude cultures following shearing of pili from the cells. A comparison of a number of approaches to this problem are described in what follows.

(a) Precipitation methods

F pili were precipitated from a culture supernatant with ammonium sulfate, polyethylene glycol 6000 (PEG 6000) in the presence of 0.5 N NaCl, or by adjusting the pH of the supernatant to 4.0, the isoelectric point of F pili.

As shown in Figure 4, the major portion of the F pili in a culture supernatant was recovered in the precipitate formed by the addition of 30% ammonium sulfate (w/v). Raising the concentration of ammonium sulfate beyond 30% recovered negligible additional amounts of precipitated F pili. Similarly 5% PEG 6000 (w/v) was the most effective concentration for precipitating F pili from culture supernatants containing 0.5 N NaCl.

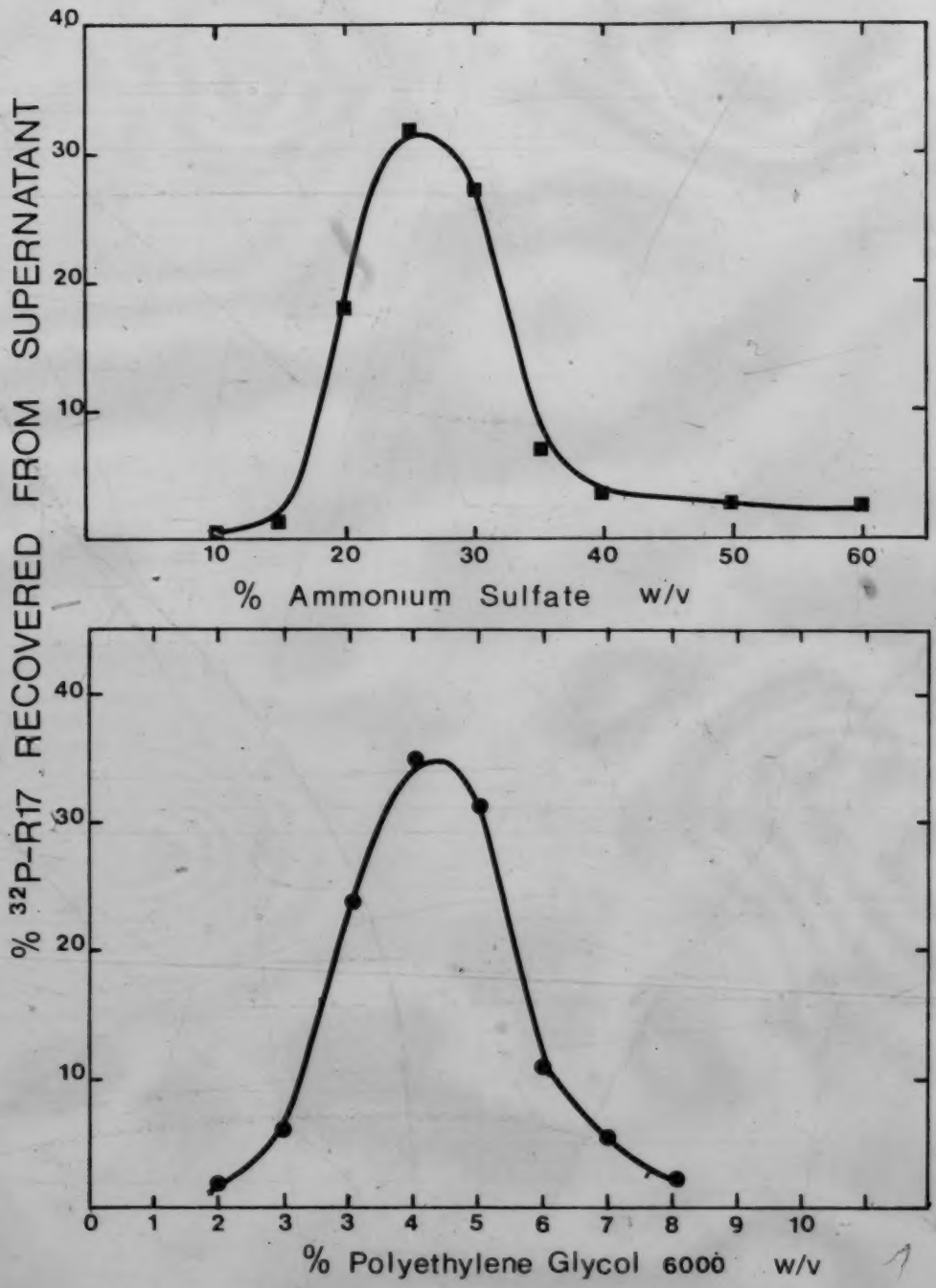
Separate 15 litre portions of a blended, F^+ culture supernatant were adjusted to pH 4.0 with 1.0 N HCl or, variously, 30% ammonium sulfate (w/v) or 5% PEG 600 + 0.5 N NaCl. The precipitates formed by these procedures were allowed to settle for 48 hours in the cold and were then harvested by low speed centrifugation.

The most efficient procedure for redissolving F pili from these precipitates was by resuspending the pelleted precipitate in

FIGURE 4. The effect of ammonium sulfate and PEG 6000 on the precipitation of *E. coli* HB11 F pili.

A 100 ml volume of a blended HB11 culture was adjusted to various concentrations of ammonium sulfate in steps of 5% from 10 to 60% (w/v). After each addition of ammonium sulfate the precipitate was allowed to settle and was collected by centrifugation. The precipitate was dissolved in 100 ml of fresh, cold medium and a ^{32}P-R17 phage attachment assay was performed on 0.5, 1.0 and 2.0 ml samples of redissolved material. The results are expressed as percent of R17 phage attachment recovered from the supernatant (■—■).

Similarly PEG 6000 was added to an HB11 culture supernatant (100 ml) containing 0.5 N NaCl in increments of 1% between 2 and 8% (w/v). The precipitate collected after each addition was dissolved in 100 ml of fresh cold medium and assayed for ^{32}P-R17 phage attachment as previously described (●-●).


% ^{32}P-R17 RECOVERED FROM SUPERNATANT
40
30
20
10
10 20 30 40 50 60
% Ammonium Sulfate w/v
40
30
20
10
0 1 2 3 3 4 5 6 7 8 9 10
% Polyethylene Glycol 6000 w/v

200 ml of SSC buffer, pH 8.5 + 10 mM urea and stirring the solution for 2 - 4 hours using a standard magnetic stirrer. The solution was then clarified by low speed centrifugation (7500 x g for 5 min) and concentrated by ultrafiltration to 20 - 30 ml. This concentration step was carried out using an Amicon 202 ultrafiltration unit (200 ml capacity) with an XM100 A filter (exclusion limit = 10^5 daltons) under a pressure of 20 psi of N_2 gas. The flow rate was approximately 80 ml/h. Overzealous stirring by the unit caused fragmentation of the F pili as visualized by electron microscopy. The relatively slow flow rate and the long exposure of the F pili to shearing forces in the unit made this procedure unacceptable for concentrating the F pili directly from the culture supernatant.

The three F pili preparations were subjected to CsCl density gradient centrifugation as described in Materials and Methods. The F pili could be visualized as a thin opalescent band midway in the gradient while other commonly found bands included a heavy band of cells and cellular debris at the bottom of the gradient, a light to heavy band of flagella at $\rho^{5°}$ = 1.32 - 1.33 depending on the strain used (HB11 or ED2687 had no such band) and a light band of membrane material at $\rho^{5°}$ = 1.20 - 1.22. A band at $\rho^{5°}$ = 1.28 represented unidentifiable debris in varying amounts which was characteristic of F pili preparations. Small amounts of F pili were enmeshed in the material in these peaks and were discarded. Typical CsCl gradient profiles for each of the three precipitation procedures are shown in Figure 5A, D and E. The F pili consistently banded at $\rho^{5°}$ = 1.26 g/cm^3. It should be noted that the other peaks in the profile are due to non-specific R17 phage retention on the filter by the material in the peaks

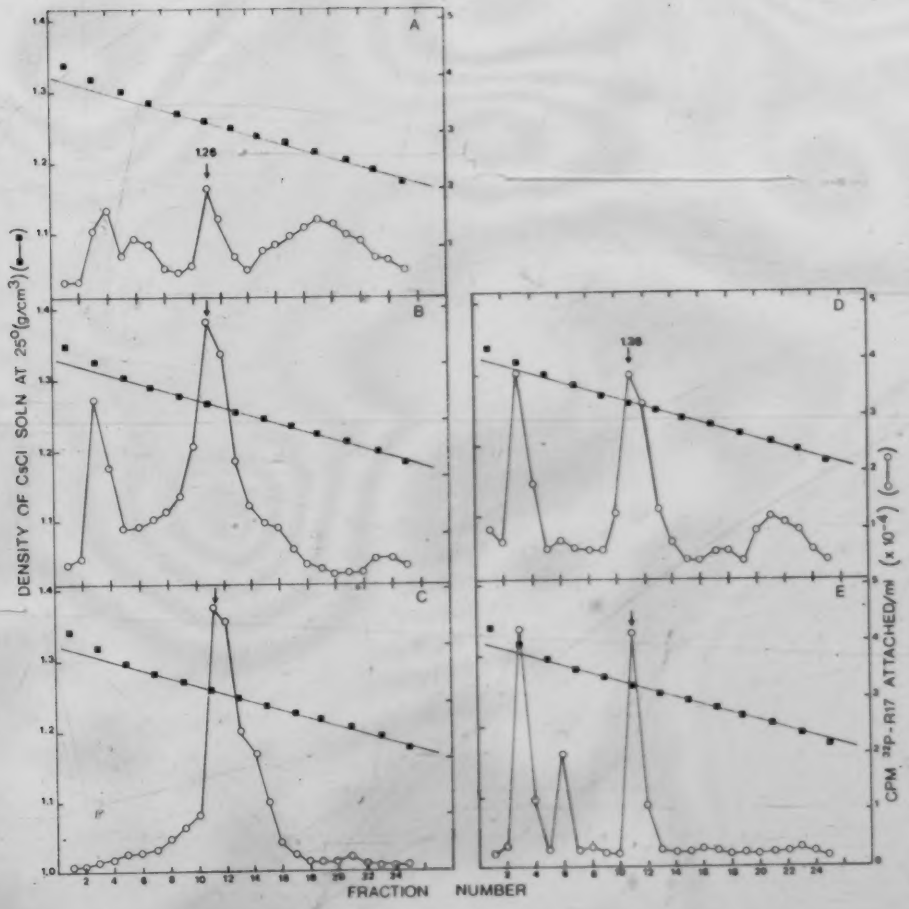


FIGURE 5. CsCl buoyant density gradient profiles of E. coli HB11 F pili prepared by various procedures.

The CsCl Gradients were formed as described in the text. The density at 5° (■-■) was calculated from the index of refraction ($n^{25°}$). A ^{32}P-R17 attachment assay (o-o) was performed on 10 µl of each fraction 0.25 ml) with 5 x 10^{11} ^{32}P-R17 particles/ml and a sample volume of 1.0 ml using the filtration assay.

The procedures used to concentrate the F pili are as follows. (A) acid precipitation at pH 4.0; (B) 30% sucrose method using cells grown on liquid minimal medium; (C) 30% sucrose method using cells grown on solid medium; (D) 30% (w/v) ammonium sulfate precipitation; (E) 5% PEG 6000, 0.5 N NaCl precipitation. The 30% sucrose method will be discussed later.

mentioned above. Electron micrographs of material taken from the F pili peak and dialyzed against SSC buffer, pH 8.5, to remove CsCl are shown in Figure 6A and B. These pictures illustrate that preparations of F pili isolated from strain HB11 contain no other extracellular appendages and that all the F pili are capable of attaching bacteriophage R17. Also they show the propensity of F pili to form huge ropes by aggregating in a side-to-side manner and trapping bits of debris in these aggregates. Surprisingly, these large ropes of F pili constituted very little protein as measured by the Lowry procedure (Lowry et al., 1951), the preparations used to produce these electron micrographs containing 1 - 10 µg/ml of protein.

(b) Two phase polymer systems

The principal difficulties in using precipitation methods with F pili are the low solubility of the precipitate and the need to use two low speed centrifugation steps, one to prepare the cell-free supernatant and one to harvest the precipitate. Since a typical purification procedure involved processing 60 litres of cells and since continuous flow centrifugation using the centrifuge available at that time caused severe fragmentation of the pili, two centrifugation steps were considered highly undesirable. Thus an alternate method was sought in order to eliminate at least one of these centrifugation steps. One such system was the partitioning of the F pili in a two-phase polymer system as described by Albertsson (1960). Separate 50 ml aliquots of cell-free supernatant were thus treated with various amounts of polymer and/or salt as shown in Table 4. The resulting two phases were carefully separated from one another and diluted to 50 ml with fresh TMM + Mg^{++}. Using a sample of the crude pili supernatant as the basis

FIGURE 6. Electron micrographs of F pili concentrated from cultures of *E. coli* HB11 and ED2602.

All specimens were negatively stained with 1% phosphotungstic acid pH 7.0 for 20 sec.

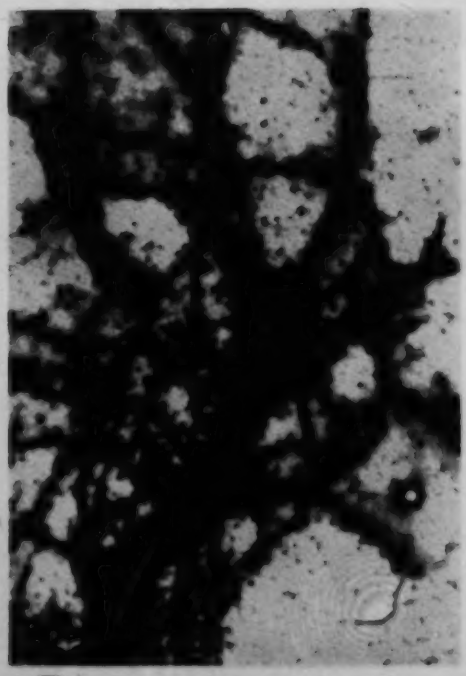
A. A 10 µg/ml solution of HB11 F pili prepared by ammonium sulfate precipitation showing "ropes" of F pili (X9000).

B. A 1 µg/ml solution of HB11 F pili labelled with 10^{14} R17 particles/ml of solution. The F pili were prepared by ammonium sulfate precipitation followed by CsCl density centrifugation (X9000).

C. A preparation of HB11 F pili with terminal "knobs" (Lawn and Meynell, 1970) caused by shearing the cells at high speeds for prolonged periods of time. These knobs represent either the base of the F pilus itself or cell wall material attached to the F pilus or the F pilus at an early stage of dissociation (X22,000).

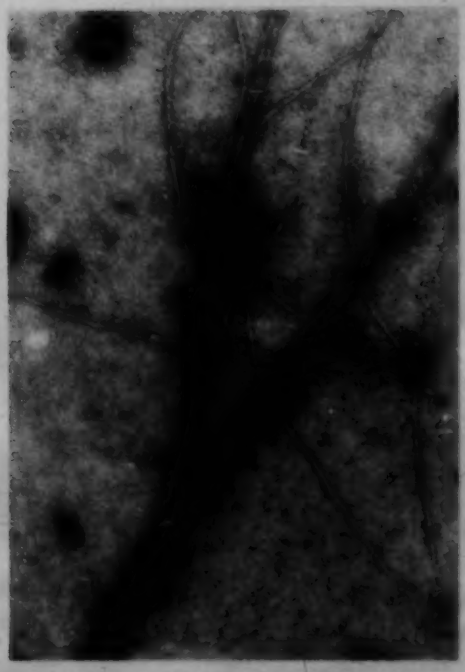
D. A preparation of ED2602 F pili (1 µg/ml) labelled with 10^{12} R17 particles/ml of solution, illustrating horseshoe-shaped vesicles found under certain conditions described in the text.

A



B

C



D

TABLE 4

The Partition of Pili in Two-Phase Polymer Systems

System	Top Phase	% Recovered	Bottom Phase	% Recovered	Interface % Recovered
1	2.5 g PEG 6000	2.8	3.0 g D2000	27.6	69.6
2	2.2 g PEG 6000	0.6	3.1 g D500	7.5	91.9
3	3.0 g PEG 6000	0.8	4.75 g D70	11.5	87.7
4	4.0 g PEG 4000	0.8	4.0 g D2000	6.8	92.5
5	4.0 g PEG 4000	0.7	4.0 g D500	4.0	95.3
6	4.0 g PEG 4000	0.7	4.0 g D70	4.6	94.8
7	1.9 g PEG 6000 + 0.3 M NaCl	0.7	3.3 g NaDS	37.0	62.4
8	2.0 g PEG 4000 + 0.3 M NaCl	2.7	3.5 g NaDS	34.0	63.4
9	4.6 g PEG 6000	9.0	6.0 g potassium phosphate	102.0	n.a.
10	4.4 g PEG 4000	27.0	6.65 g potassium phosphate	78.0	n.a.

Abbreviations: PEG - polyethylene glycol; D - dextran; NaDs = sodium dextran sulfate.

A crude F pili supernatant was divided into 50 ml portions and partitioned by the above two-phase polymer systems as described in the text. The top and bottom phases as well as the interface were separated from one another and diluted to 50 ml. Samples (0.5, 1.0 and 2.0 ml) from each phase were assayed for ^{32}P-R17 phage attachment using 10^{11} particles/sample. This procedure was repeated for each system using water instead of the crude F pili preparation to determine the amount of non-specific attachment contributed by the polymers themselves. This attachment was negligible after the filters had been washed with TMM + Mg^{++}.

for comparison, a ^{32}P-R17 phage attachment assay was done on 0.5, 1.0 and 2.0 ml samples from each phase and the results are reported in terms of percent recovered from the crude supernatant (Table 4).

The F pili were found concentrated at the interface and to a lesser degree in the bottom phase of systems 1 - 8. The polyethylene glycol-potassium phosphate systems had no precipitate at the interface. Systems 5, 6 and 9 were scaled up to 2 litres and the ratio of volume of the top phase to the volume of the bottom phase was adjusted to 10:1 using the phase diagrams provided by Albertsson (1960). Again the results were the same with the F pili concentrated at the interface and the bottom phase. Unfortunately, two-phase systems are practical only for concentrating small amounts of material because of the high cost for large volumes of supernatant. Attempts were also made to test the two-phase system (PEG 6000:potassium phosphate) for concentrating redissolved F pili precipitates as an alternative to ultrafiltration (see above section). However, because cellular debris partitioned into the same phase as the F pili and the two-phase method required additional steps to remove the polymer from the concentrated pilus solution, ultrafiltration remained the method of choice for further work.

(c) Sucrose-dependent procedures

In 1971, Brinton stated that high-speed blending of cultures caused bits of the cell membrane to be torn off the cell along with the base of the F pilus. This would contribute to the problem of cellular debris contamination. He recommended that the cells be collected by centrifugation prior to blending, after which gentle stirring in 30% sucrose was supposed to achieve the removal of F pili from cells and promote solubility of F pili as well as prevent the readsorption of the

F pili to the cells. The cells were pelleted by centrifugation and the sucrose was removed from the supernatant by extensive dialysis during the course of which the F pili aggregated into large ropes or "crystals" which could be pelleted by low speed centrifugation. This procedure was found to be far less time-consuming than the precipitation methods. It also avoided a precipitation step which lowered the solubility of the F pili. Thus it was adopted for this investigation.

Typically, a 15 litre culture of HB11 similar to the one previously described was treated with formaldehyde and chilled in ice water. The cells were harvested by low speed centrifugation and the supernatant was discarded. The cells were resuspended in 200 ml of 30% sucrose in SSC buffer, pH 8.5, and gently stirred using a Fisher magnetic stirrer overnight in the cold. The cells were removed by centrifugation at 10000 x g for 20 min to give a translucent supernatant. The supernatant, containing the F pili, was dialyzed for 96 h against SSC buffer, pH 8.5, with frequent buffer changes which caused the F pili to form large ropes. These ropes of F pili were pelleted by low speed centrifugation at 27000 x g for 1 h. The pili were re-dissolved in 27 ml of SSC buffer, pH 8.5, and banded on a CsCl gradient as previously described. The CsCl gradient profile is shown in Figure 5B. This procedure resulted in the best yields of F pili achieved up to that time (0.5 - 1.0 mg from 15 litres of culture) and was generally an improvement in time and effort required.

However, since F^+ cells grown on solid media (TSB agar) produced an equivalent number of F pili/cell as cells grown in liquid media, it seemed feasible to harvest cells from large sheets of agar and thus entirely eliminate the tedious steps of blending the cells and centrifuging large volumes of culture.

Briefly, sterile aluminum trays (27 x 38 x 1.25 cm) containing approximately 1 cm of TSB agar (350 ml) were spread with 10 ml of a culture of HB11 cells (5 x 10^8 cells/ml) grown in TSB broth. The trays were covered and incubated at 37°C for 18 h. The cells were scraped off the surface of the agar with a 10 cm putty knife and resuspended in 30% sucrose in SSC buffer, pH 8.5. Routinely, 36 trays were prepared each time, usually three times per week. Thirty-six trays yielded approximately 250 g (wet weight) of cells which was approximately the equivalent of 6 carboys containing 15 litres of cells at 1 x 10^9 cells/ml. The cells, suspended in 500 ml of 30% sucrose, were passed through a fine sieve to remove bits of agar and were stirred with a magnetic stirrer overnight in the cold. The cells were removed by centrifugation and the supernatant was handled as previously described for F pili separated from cells grown in liquid culture (see Figure 5C).

The disconcerting production of horseshoe-shaped vesicles (see Figure 6D) by cells carrying the plasmid JCFLO (e.g. ED2602 and ED2687) was thought to be due to the disintegration of the F pili during stationary phase when the cells were grown on solid media. These vesicles, while incapable of R17 phage attachment, behaved identically to F pili in CsCl density gradients, isoelectric focusing and SDS gel electrophoresis (to be described in the next chapter). This was the only disadvantage to a system which was far simpler to use than any other described here.

With the exception of acid precipitation, the yield of F pili from the procedures reported in Figure 5 was approximately 0.3 - 0.5 mg from 15 litres of culture (~40 g (wet weight) of cells) as determined by the Lowry assay for protein (Lowry et al., 1951). Acid

precipitation of F pili at pH 4.0 gave only 100 µg of protein from a similar volume of culture. As seen in Figure 7, electropherograms of SDS polyacrylamide gels of ED2687 F pili concentrated using the 30% sucrose method reveal that the preparation is contaminated with high molecular weight material that is largely removed by a CsCl density gradient step. The double peak at 2 - 3 cm was characteristic of the contamination in F pili preparations. Comparing the amount of ^{32}P-R17 phage attachment in the whole purified F pilus preparation using the filtration assay to the attachment capacity for the total volume of culture supernatant prior to the purification procedure, only 30 - 40% of the total R17 phage attachment capacity was recovered in the purified F pili. While the amount of protein was quite similar regardless of procedure, the amount of carbohydrate material in these preparations as indicated by the orcinol colorimetric assay (Ashwell, 1957) varied enormously dedepending on the procedure used. Figure 8 shows a composite of several paper chromatograms, comparing the sugar content of F pili concentrates prepared by the procedures described above. One to 2 mg of protein was hydrolyzed in 2 N HNO_3 in a boiling water bath for five hours. The hydrolysate was dried in an oven at 110°C and was resuspended in 0.1 ml of 50% ethanol. The sample was applied with a 5 µl micropipette at the origin located 10 cm from one end of a sheet of Whatman 1 MM paper, 23 x 56 cm. Standard sugars (25 µg) were also applied adjacent to the hydrolysate. The chromatogram was then subjected to liquid chromatography as described in Materials and Methods. It may be seen that the F pili prepared by acid precipitation contained a wide assortment of sugars as described by Donelian (1972). The other procedures were an improvement over the acid precipitation procedure in terms

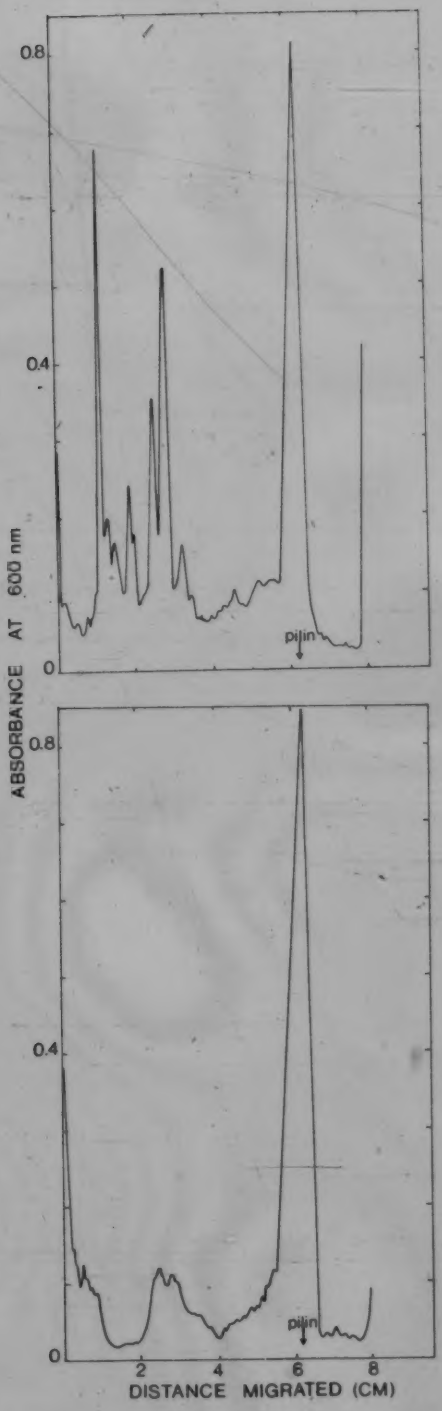
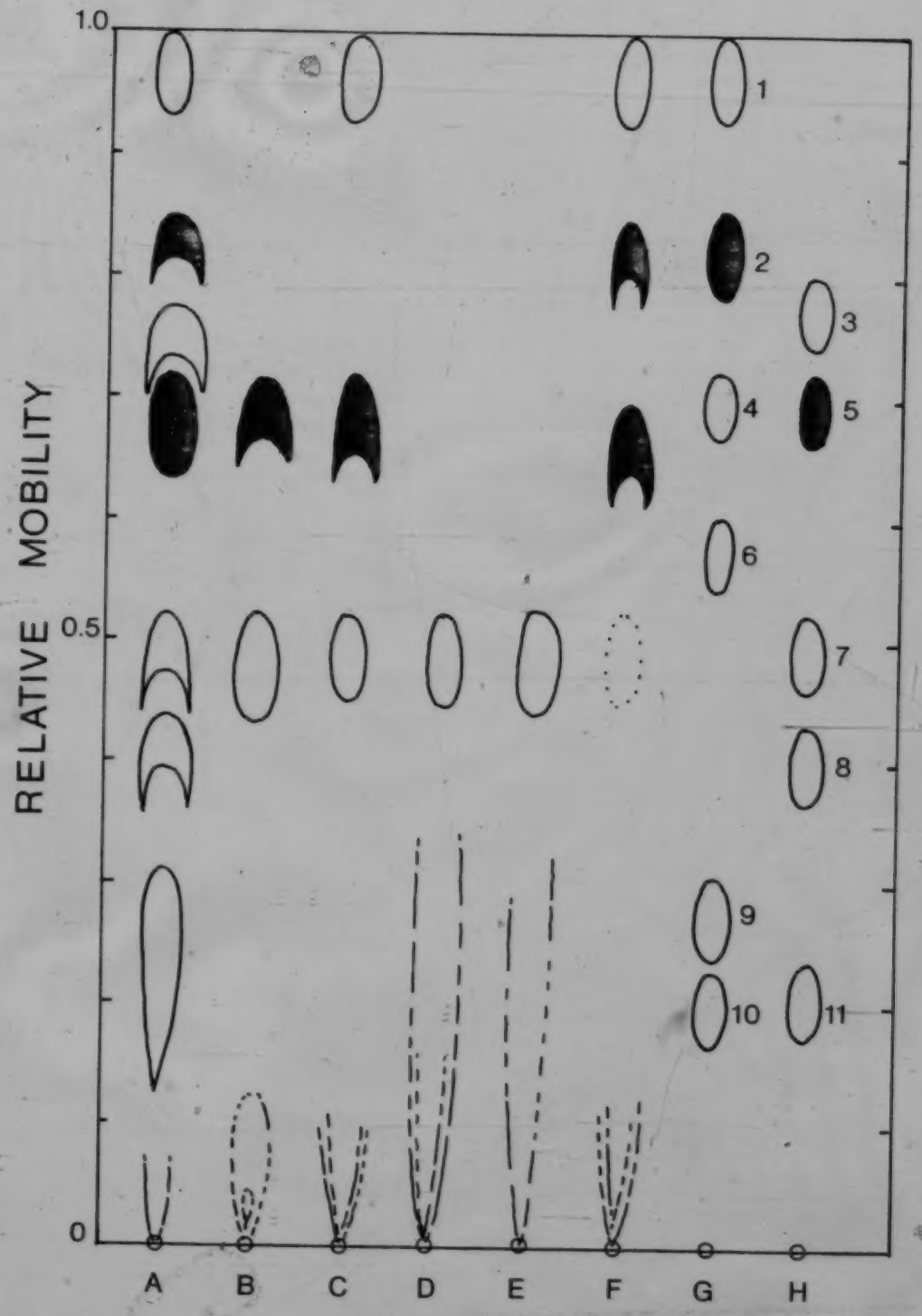


FIGURE 7. Electropherograms of SDS polyacrylamide gels of E. coli ED2687 pilin.

SDS polyacrylamide disc gel electrophoresis was performed using 30 µg of protein taken from a preparation of ED2687 F pili before (top panel) and after (bottom panel) CsCl density centrifugation. The gels were overloaded with F pili in order to visualize the heavier molecular weight bands in the gels. The stained gels were scanned at 600 nm using a Gilford recording spectrophotometer with a gel scanning attachment.

FIGURE 8. Paper chromatogram of carbohydrate material found in various F pilus preparations.

The relative mobilities of each spot were calculated with respect to rhamnose. The standard compounds in positions G and H represent 15 µg of material each: (1) rhamnose; (2) 3,1-dideoxyglucose; (3) ribose; (4) N-acetylglucosamine; (5) 3-deoxyglucose; (6) arabinose; (7) glucose; (8) galactose; (9) glucosamine; (10) galactosamine; (11) lactose. The unusual sugars were the gift of Dr. R.U. Lemieux. The pili preparations containing 1 - 2 mg of protein were purified as follows: A, acid precipitation, pH 4.0; B, ammonium sulfate precipitation; C, PEG 6000, 0.5 N NaCl precipitation; D, 30% sucrose method liquid culture medium; E, 30% sucrose, solid culture medium; F, HB11 F⁻ "pili" preparation.


1.0
0.5
0
RELATIVE MOBILITY
1
2
3
4
5
6
7
8
9
10
11
A
B
C
D
E
F
G
H

of removing carbohydrate contamination. The F pili prepared without blending, i.e. by stirring in sucrose, gave no spots other than glucose and some faint trailing from the origin indicating that blending may give rise to membrane material, the most likely source of carbohydrate contamination. Characteristically late-developing spots, first noted by Donelian (1972), were identified as deoxy- and dideoxy-hexose compounds, components of the E. coli membrane. The only sugar present in all hydrolysates was D-glucose in a ratio of 1 - 2 molecules of D-glucose/F pilin subunit, using the size of the spot to estimate the amount of glucose present and based on a molecular mass for F pilin of 12,000 daltons. A preparation of "F pili" using HB11 F^- cells and acid precipitation at pH 4.0, contained the deoxy- and dideoxy-sugars, and an unidentified pentose but had a less intense glucose spot (<5 µg compared to 10 - 20 µg for F^+ preparations).

C. CONCLUSION

On the basis of the foregoing studies, it has been found that the purification of F pili can be accomplished most easily by removing the pili from a concentrated solution of cells in the presence of 30% sucrose. This minimizes the amount of cellular and membrane debris in the F pilus preparation. After removal of the cells by centrifugation, and the sucrose by dialysis, the F pili can be pelleted and further purified by CsCl gradient centrifugation. The yield was found to be 1 - 2 mg of F pili per 100 g wet weight of cells for the bacterial strains used here (HB11, ED2687).

This is essentially the method reported by Minkley et al. (1976) who obtained 5 mg of pure F pili from 24 litres of culture. Their high-

er yield can be attributed to their use of a slightly multi-piliated traD mutant bacterial strain. Date et al. (1978) also used essentially this same method but included a 25 - 70% (w/v) sucrose gradient step prior to CsCl gradient centrifugation. Their yield of 1.5 - 2.0 mg of F pili from 67 g (wet weight) is similar to the findings in this investigation. Also, they used a solid medium to culture their cells and achieved yields of F pili comparable to that of workers using liquid media.

The sucrose-based method is also useful in the purification of other types of pili. Besides using it in the purification of P. aeruginosa K pili (see Chapter V and VI) it has been used in the purification of pili from N. gonorrhoeae (Robertson et al., 1977). Two other reports on amino acid composition and N-terminal sequence of pilin used blended cells followed by precipitation with methanol in the cold (M. nonliquefaciens pili, Frøholm and Sletten, 1977) or ammonium sulfate (10% saturation) precipitation (N. gonorrhoeae, Hermodson et al., 1978).

Thus while several methods including that of Beard et al. (1972b), which used ultrafiltration and isoelectric focusing to concentrate and purify F-type R pili, have been used successfully to prepare relatively pure F pili, the yield of F pili using any of the bacterial strains studied to date is always fairly small, ranging from 1 - 5 mg of pure material from approximately 40 - 60 g of cells (wet weight).

Several pitfalls in the purification of pili should be mentioned at this time. Firstly, blending cells to remove the pili contributes to the presence of vesicle-like material in the preparation. This is most likely derived from the membrane of the cell. Stirring the cells in 30% sucrose gave the least amount of this contamination as determined

by electron microscopy and carbohydrate analysis.

Growing the cells on solid medium in large pans can be used to give good yields of pili if care is taken to maintain the temperature of the pans of agar at 37° after they have been inoculated with the cells. Also, to prevent the disintegration of the F pili into small vesicle-shaped fragments, the cells must not be allowed to remain in stationary phase too long. Treating the cultures with formaldehyde and chilling them as quickly as possible seems to help maintain the levels of F pili in the culture. Allowing the cells to cool to room temperature without poisoning them results in F pilus loss as noted by Novotny and Lavin (1971).

Precipitation of the F pili to effect concentration from the medium, during the purification procedure, leads to a rather insoluble precipitate. The precipitating agents tried by this author resulted in the co-precipitation with the F pili of large amounts of carbohydrate and protein material which were extremely difficult to separate from the F pili. This occurred regardless of whether a rich or synthetic medium was used although broth contributed much more of this material than synthetic medium. This problem had been noted previously by Brinton and Beer (1967), Valentine et al. (1969) and Donelian (1972). Finally, while CsCl density centrifugation was a valuable tool for separating F pili from cellular debris, long-term exposure of the F pili to CsCl caused a loss of pilus material. This had previously been noted by Valentine et al. (1969). Because of this, a maximum of one CsCl gradient was employed in the purification of F pili in the latter stages of this investigation. Sucrose gradients and Cs_2SO_4 density gradients are now used extensively in this lab to avoid unnecessary loss of material.

The small amounts of F pili in a bacterial culture have been the biggest obstacle to significant advances in the study of the chemistry of F pili. While interest has been high for over a decade and the capability to purify F pili sufficiently has been known for at least five years, preliminary reports of chemical studies on F pili have only recently begun to appear in the literature (Date et al., 1978). No reports have yet appeared in which amino acid sequence data are provided presumably because amino acid sequence studies require substantial amounts of material, i.e., approximately 100 - 200 mg would be required to establish the complete sequence of a protein such as F pilin. Since nearly 3000 litres of culture would be required to produce this amount of F pili and because this volume of cells would be both costly and time-consuming to process, progress has been very slow.

CHAPTER IV

PHYSICOCHEMICAL PROPERTIES OF F PILI

A. INTRODUCTION

While the ultimate objective of a chemical analysis of F pili was never reached, several physical and chemical properties of F pili were studied including the buoyant density in CsCl, the isoelectric point, the molecular weight as determined by SDS disc gel electrophoresis, and the carbohydrate content as discussed in the previous chapter. In addition, F pili were successfully labelled with ^{32}P and ^{35}S and evidence that F pili contain a covalently-linked phosphate group was obtained.

The studies presented here used ED2687 or HB11 F pili isolated from cells grown on trays of agar as described in Chapter III.

B. RESULTS AND DISCUSSION

1. CsCl buoyant density gradient centrifugation

The density of F pili in CsCl density gradients appears to vary depending on the plasmid and/or the host-strain used to produce the F pili (Beard, cited by Tomoeda et al., 1975). The density of F pili has been determined as 1.257 (Brinton, 1971), 1.296 (Beard et al., 1972a), 1.197 (Wendt et al., 1966) and 1.232 (Tomoeda et al.; 1975, Date et al. 1978), while F-like R pili have a density of 1.31 (Beard et al., 1972a) and gonococcal pili from N. gonorrhoeae have a density of 1.30 to 1.31 (Robertson et al., 1977).

Beard (1973) has reported that F pili specified by two independently maintained Flac plasmids in two strains of E. coli K12 induced "heavy" (ρ = 1.296) and "light" (ρ = 1.257) F pili in another strain.

He also found that one Flac plasmid produced small amounts of pili at the alternate density while the other plasmid did not.

Essentially similar findings were found by this author for F pili derived from three different F plasmids and F-like R pili from three different R plasmids. These results are presented in Figure 9.

It may be seen that F pili encoded by the F plasmid, HBFLO, banded in CsCl at a density of 1.26 g/cm^3, while the plasmid JCFLO produced F pili which banded predominantly at 1.26 but also contained a small amount of material which banded at density 1.30. Neither the host strain (ED2601 for ED2602 and ED2612 or HB11 F^- for ED2687) nor a traD8 mutation in JCFLO (ED2612) altered this general distribution of F pili in the gradient. The F plasmid UBFLO (E. coli K12, strain UB1105), used by Beard in his studies (Beard et al., 1972a) and kindly donated by him for comparison to the other F plasmids, gave a single band at density 1.30. The derepressed F plasmids R100-1, R1-19 and R538-1 in host strain ED2601 (ED2603, ED2604 and WP128) gave only "heavy" pili at a density of 1.31 which is in accordance with Beard's findings. All bands of pili were re-banded in CsCl and were found at the same density as in the original CsCl gradient.

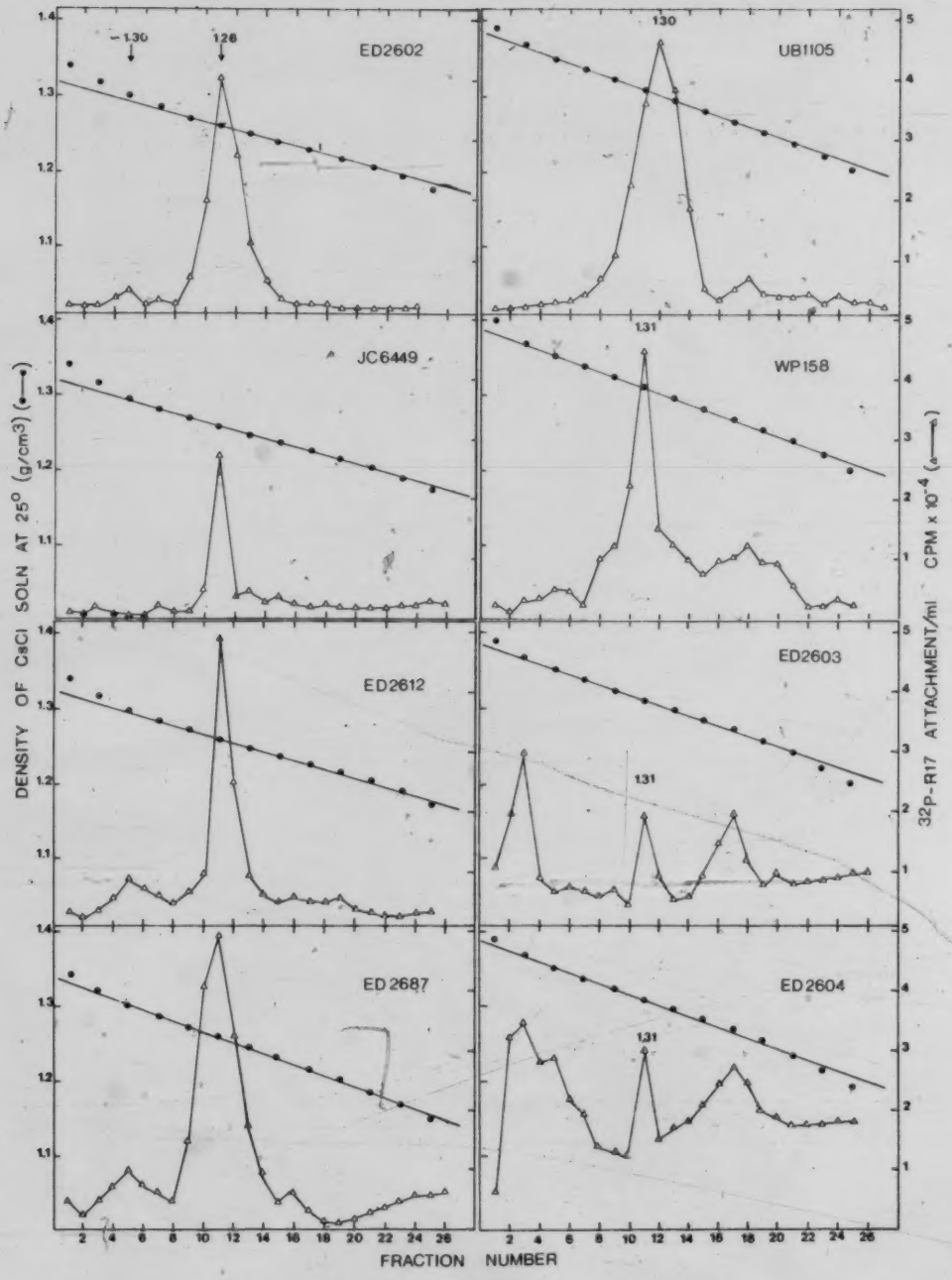
The small amount of material at the higher density (1.30) may represent a modified version, in vivo, of the lighter F pili, or it may be an artifact caused by the presence of CsCl.

2. Isoelectric focusing of F pili

The previously reported isoelectric point values for pili include pH 4.15 for F pili (Brinton, 1971), pH 3.5 for F-like R pili (Beard et al., 1972b), pH 3.9 for type 1 pili (Brinton, 1965) and pH 4.9 and 5.3 for gonococcal pili (Robertson et al., 1977).

FIGURE 9. Profiles of CsCl gradients of F pili isolated from eight different strains of *E. coli*.

The F pili produced by each strain was purified from cells (approximately 125 g (wet weight)) followed by treatment with 30% sucrose to separate the F pili from the cells. The conditions for CsCl isopycnic centrifugation are described in Chapter II. A 50 µl sample from each fraction of the CsCl gradient was assayed for ^{32}P-R17 phage attachment using the filtration assay (Δ-Δ). Density of CsCl solution (•-•). ED2602 = JCFLO/ED2601; JC6449 - JCFL*traD8*/JD3272; ED2612 = JCFL*traD8*/ED2601; ED2687 - JCFLO/HB11 F⁻; UB1105 = UBFLO/UB1025 F⁻; WP128 = R538-1/HB11 F⁻; ED2603 = R100-1/ED2601; ED2604 = R1-19/ED2601.


ED2602
UB1105
JC6449
WP158
ED2612
ED2603
ED2687
ED2604
130
126
131
DENSITY OF CsCl SOLN AT 25° (g/cm3) (•—•)
32P-R17 ATTACHMENT/ml CPM x 10⁻⁴ (▵——▵)
FRACTION NUMBER

F pili, purified by two rounds of CsCl density centrifugation, were dialyzed exhaustively against 1% glycine (w/v). The procedure for isoelectric focusing (Vesterberg and Svensson, 1966; Beard et al., 1972b) is described in Materials and Methods. The column (110 ml LKB 8101 column) contained 2 mg of protein and 5% ampholyte (LKB ampholine) pH range 3.5 - 10.

A voltage of 350 volts (10 ma) was applied for 24 hours at which time the current had dropped to <2 ma. The bands were sharpened by increasing the voltage to 450 volts for five hours or until the bands were distinct.

Two strains were used to give the pI of F pili, HB11 and ED2687 (Figure 10). The presence of F pili was assayed by ^{32}P-R17 phage attachment and EM visualization. The pI of ED2687 pili was pH 4.0 while HB11 F pili had a pI of 3.9. Secondary peaks at pH 4.8 and 6.4 in the ED2687 pH gradient contained small amounts of F pili but were predominantly vesicle-like material. SDS-polyacrylamide gel electrophoresis of material from the major peak at pH 3.9 or 4.0 revealed that high molecular weight contaminants had co-banded with the F pili. Thus isoelectric focusing was not effective in reducing contamination. Similar results have recently been published by Date et al. (1978).

3. SDS-Polyacrylamide gel electrophoresis

The molecular weight for pilin, the subunit of pili, has been reported on the basis of SDS gel electrophoresis to be 11,800 for F pili (Brinton, 1971; Date et al., 1978), 12,500 for F-like R pili (Beard and Connally, 1974), 17,000 for type 1 pili (Brinton, 1965) and 18,000 to 20,000 for gonococcal pili from several strains (Robertson et al.,

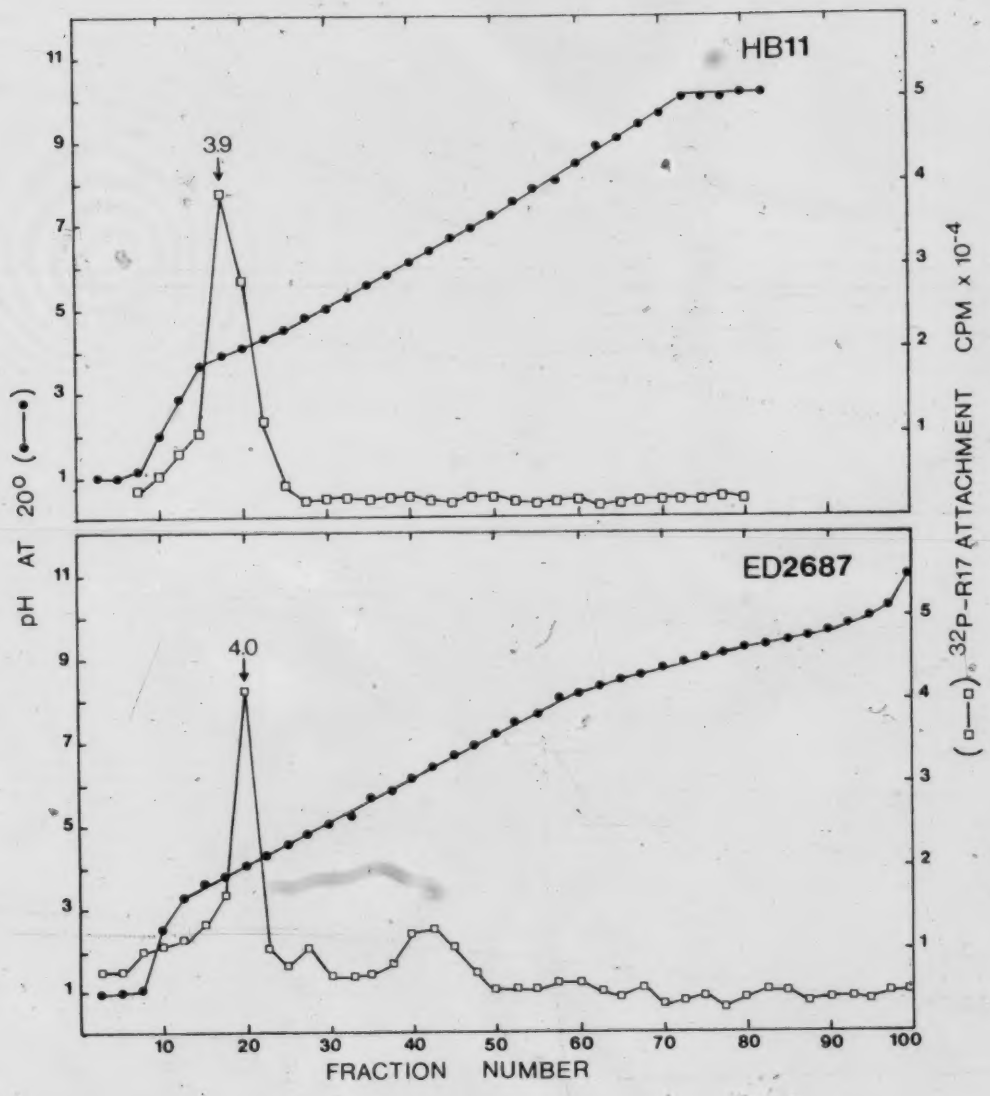


FIGURE 10. Isoelectric focusing of E. coli HB11 and ED2687 F pili..

A 1.5 - 2.0 mg of F pilus material was banded in a 110 ml linear sucrose gradient containing 2% ampholytes (w/v) pH range 3.5 to 10 at 10° with an average voltage of 400 V as described in Chapter II. A 50 µl portion of each fraction was assayed for ^{32}P-R17 phage attachment using the filtration assay (□-□). pH (●-●).

1977).

SDS polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborne (1969) and the gels were stained using a modified procedure of Fairbanks et al. (1971), as described in Materials and Methods.

Using the standards listed in Figure 11 to construct a curve relating relative mobility to molecular weight, the molecular weight was calculated as 11,900 ±400 and was the same for F pili derived from HB11, ED2687 and the F-type R plasmid R1-19 (12,500).

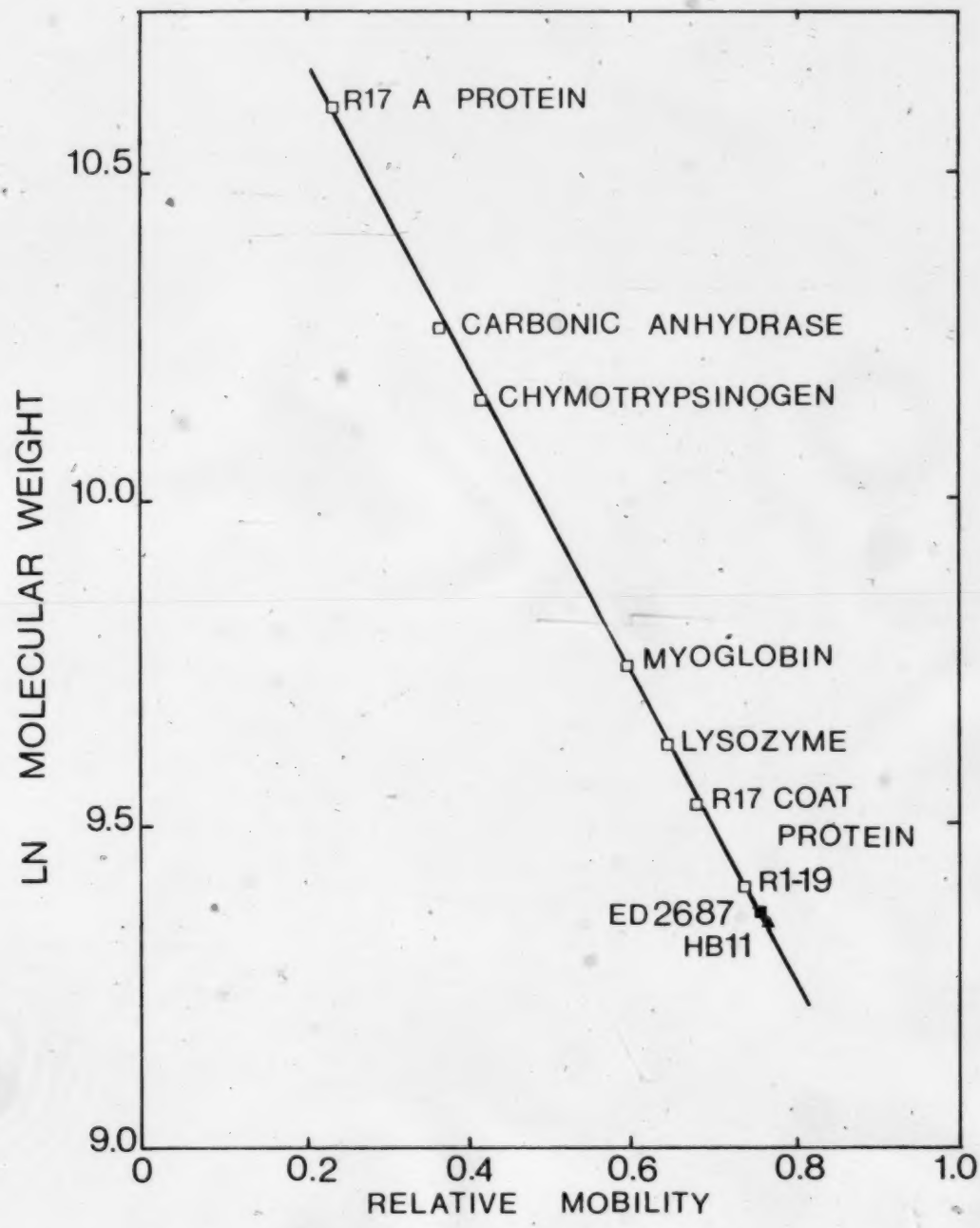
4. Studies with radioactively-labelled F pili

Brinton (1971) has reported that one F pilin monomer contains one molecule of D-glucose and two phosphate groups covalently linked to the pilin protein. Beard and Connally (1974) have stated that R1drd19 pilin contains one each of glucosamine and galactose as well as one molecule of D-glucose and two phosphate groups. Date et al. (1978) have also detected carbohydate and phosphate in F pili. Robertson et al. (1977) have shown that gonococcal pili also contain 1 - 2 phosphate groups, galactose and some glucose depending on the strain of N. gonococcus being studied. Several studies (Brinton, 1971; Donelian, 1972; Beard et al., 1972b; Robertson et al., 1977) have shown that pili contain no lipid nor nucleic acid material.

A study employing radioactive ^{32}P-labelled F pili was undertaken to determine whether the phosphate was covalently bound to the F pilin or was merely a contaminant arising from membrane debris. ^{32}P-labelled F pili were prepared as follows. One liter of lacCTMM + aa containing one tenth the usual amount of phosphate (0.5 µM Na_2HPO_4) plus 10 mCi ^{32}P (as carrier-free phosphoric

FIGURE 11. Molecular weight determination of F pilin from *E. coli* HB11 and ED2687 using SDS polyacrylamide gel electrophoresis.

SDS disc gel electrophoresis was performed according to the method of Weber and Osborn (1969). A 10 - 15 µg amount of standard protein or F pilin was run on a 10 cm gel containing 12.5% acrylamide, 0.34% N,N'-methylenebisacrylamide in 0.05 M sodium phosphate buffer, pH 7.2, 0.1% SDS, at 5 ma/gel until the marker dye had migrated 8 cm down the gel. The molecular weight of the standards was taken as that reported by Weber and Osborn (1969). The R1-19 pili (MW = 12,000) was kindly donated by Dr. J.P. Beard.


R17 A PROTEIN
CARBONIC ANHYDRASE
CHYMOTRYPSINOGEN
MYOGLOBIN
LYSOZYME
R17 COAT PROTEIN
R1-19
ED2687
HB11
LN MOLECULAR WEIGHT
10.5
10.0
9.5
9.0
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

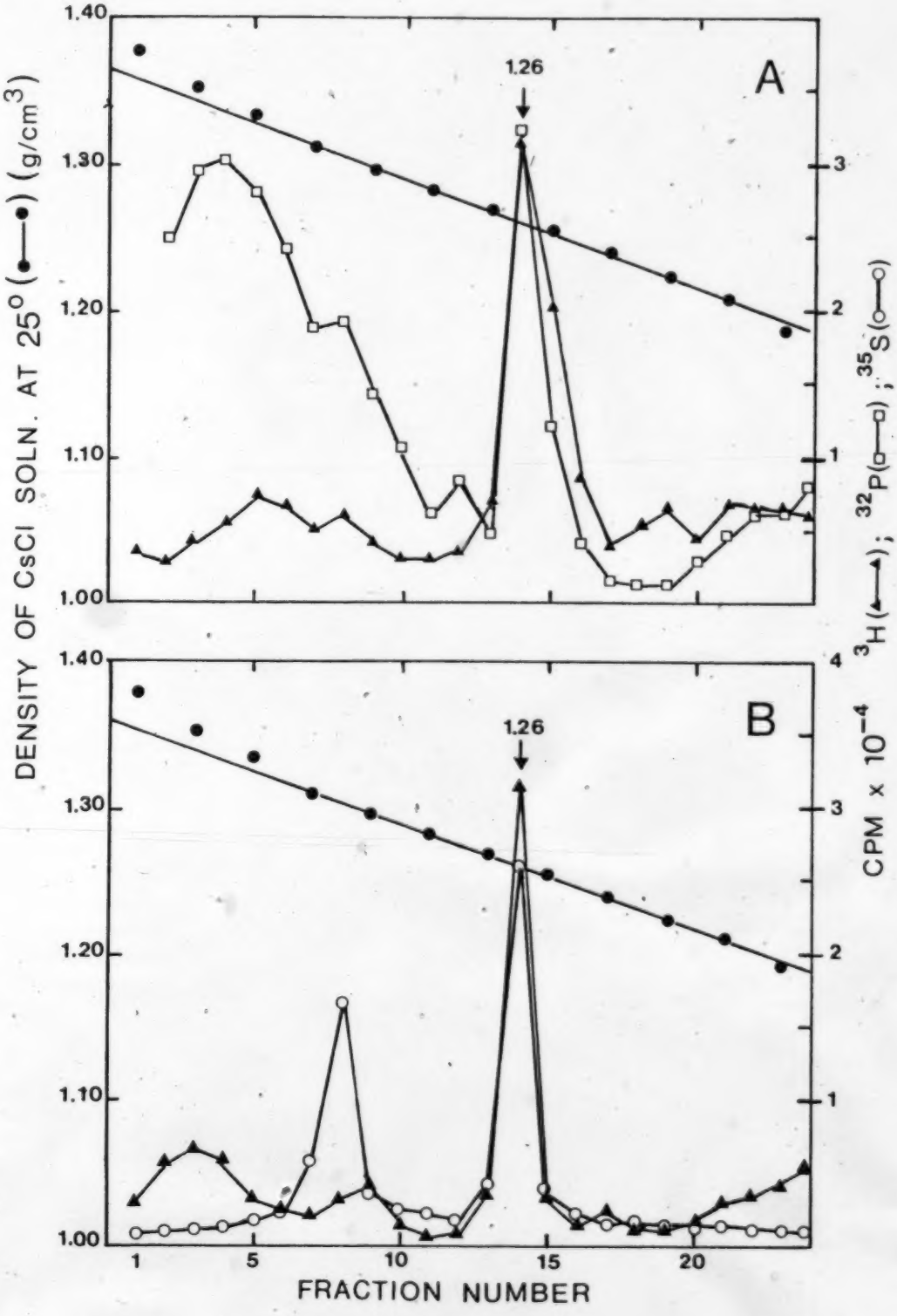
acid in water, neutralized with 0.1 N NaOH) was pre-warmed to 37° and inoculated with 10 ml of fresh standing overnight culture of ED2687 grown to 0.4 A_{650} units in reduced phosphate minimal medium. The culture was grown in a Brunswick shaking water bath to 1 x 10^9 cells/ml (1.7 A_{650} units). The culture was treated with 4.0 ml of formalin, chilled in ice water and the cells were removed by low speed centrifugation at 10000 x g for one hour. The cells were resuspended in 30 ml of 30% sucrose in SSC buffer, pH 8.5, and then gently stirred for three hours in the cold. The cells were removed by low speed centrifugation at 10000 x g for 15 min and the supernatant was dialyzed for three days against 6 x 2 litres SSC buffer, pH 8.5. The F pili were pelleted at 27,000 x g for 90 min and the pellet was resuspended in 4.5 ml of SSC buffer, pH 8.5. The pili were banded at $\rho^{5°}$ = 1.26 g/cm^3 in a CsCl gradient and 50 µl from each fraction of the gradient was assayed for R17 phage attachment using ^{3}H-guanosine R17.

The profile of this CsCl gradient is given in Figure 12A. In addition to the large F pili peak at $\rho^{5°}$ = 1.26 g/cm^3 labelled with ^{32}P the characteristic smaller pili peak at $\rho^{5°}$ = 1.30 g/cm^3 was also labelled with ^{32}P. The presence of F pili at these densities was confirmed by electron microscopy.

The possibility existed that the ^{32}P found at $\rho^{5°}$ = 1.26 g/cm^3 (see Figure 12A) was associated with cellular or membrane material which had co-banded with the F pili. To examine this possibility, ^{3}H-labelled R17 was attached to F pili labelled with ^{32}P and these complexes were banded in a CsCl density gradient. R17 particles, by themselves, band at $\rho^{5°}$ = 1.44 g/cm^3 (Krahn et al., 1970) while F pili band at $\rho^{5°}$ = 1.26 g/cm^3. R17 - F pili complexes band at an intermediate density

FIGURE 12. CsCl density gradients of *E. coli* ED2687 F pili labelled with radioactive ^{32}P or ^{35}S.

(A) ^{32}P-labelled F pili banded in a CsCl gradient (□-□).
(B) ^{35}S-labelled F pili in a CsCl gradient (o-o).
A 50 µl sample of each fraction was assayed for R17 phage attachment using ^{3}H-guanosine-labelled R17 (▲-▲).


A
B
1.26
1.26
1.40
1.30
1.20
1.10
1.00
DENSITY OF CsCl SOLN. AT 25° (●—●) (g/cm³)
CPM x 10⁻⁴ ³H(▲—▲); ³²P(□—□); ³⁵S(○—○)
1
2
3
4
1
5
10
15
20
FRACTION NUMBER

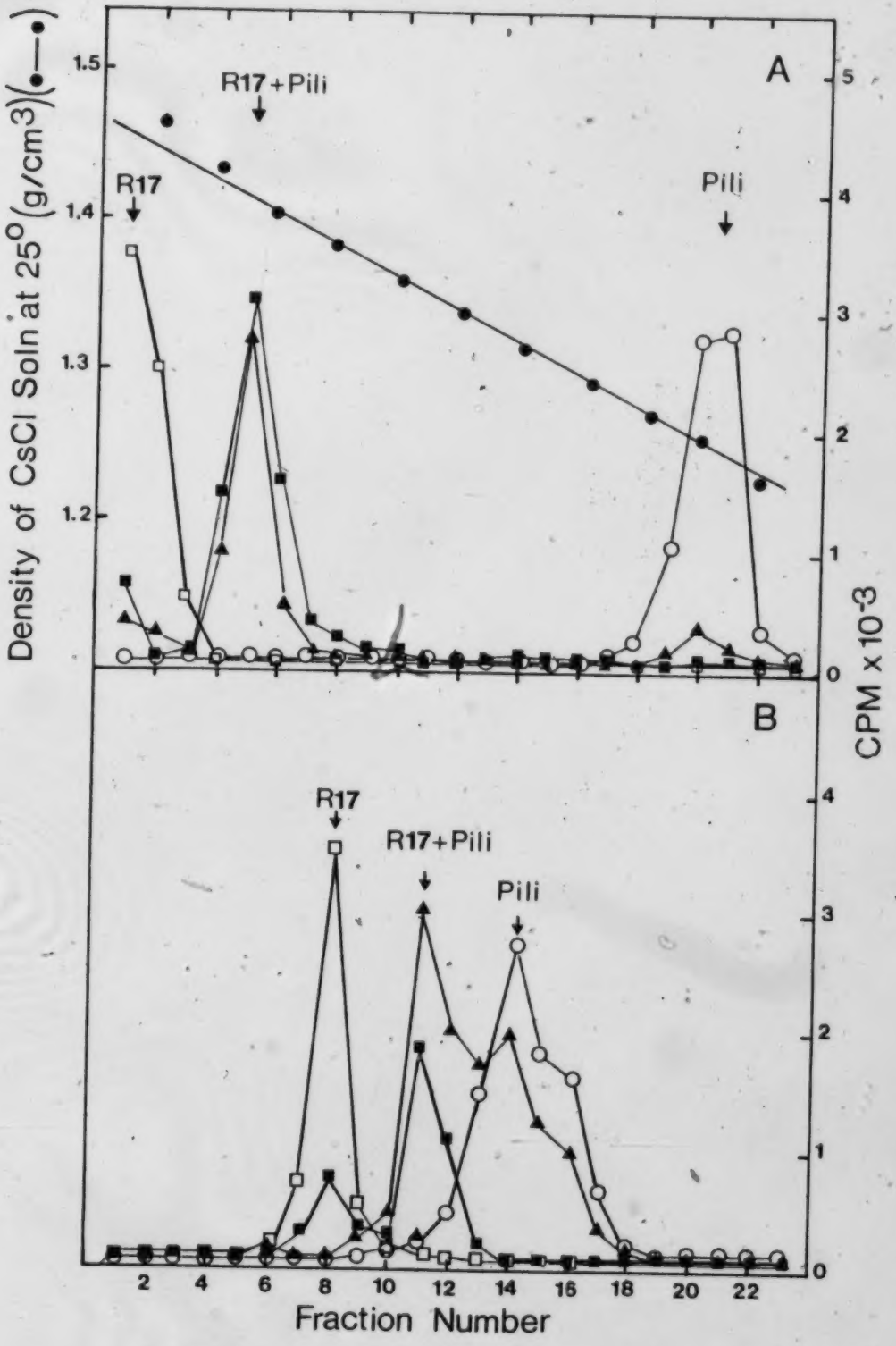
(Beard et al., 1972). ^{32}P-associated with the F pili should band at the intermediate density while ^{32}P associated with cellular or membrane debris should band at the top of the gradient at $\rho^{5°} \leq 1.26$ g/cm^3. A sample containing 0.2 ml of ^{32}P-labelled F pili (5000 cpm) and 1 µl of ^{3}H-G-R17 (50,000 cpm/7.0 x 10^{14} p) previously incubated in the presence of 5 mM $MgCl_2$ and fixed with 1% formaldehyde was banded in a CsCl gradient (median density 1.38 g/cm^3) containing 5 mM $MgCl_2$ as previously described. A 100 µl portion of each fraction was assayed for ^{3}H and ^{32}P radioactivity and the appropriate corrections for overflow were made as described in Chapter II. Identical CsCl gradients were run containing only ^{32}P-labelled F pili or ^{3}H-labelled R17. The remaining portion of each fraction was used to establish its density at 5°. The contents of each peak were visualized by electron microscopy. The results are given in Figure 13A. R17 - F pilus complexes were found at $\rho^{5°} = 1.42$ g/cm^3 while free R17 banded at 1.44 g/cm^3 and free F pili floated at the top of the gradient. A small portion of the ^{32}P counts (10%) was found at the top of the gradient, unassociated with ^{3}H-R17. Since very little F pilus material was found at the latter position, this peak was taken to represent either low density cellular debris, free ^{32}P, or dissociated pili.

Similarly, identical samples of either ^{32}P-F pili complexed with ^{3}H-R17 or free ^{32}P-F pili or free ^{3}H-R17 were layered on a 5.0 ml 5 - 20% (w/v) continuous sucrose gradient in SSC, pH 8.5, containing 5 mM $MgCl_2$. The gradient was centrifuged at 45,000 rpm in a SW 50.1 rotor at 5° for 60 min and fractions were collected as previously described. The fractions were assayed for ^{3}H and ^{32}P radioactivity and the nature of the peaks in each gradient was checked using electron microscopy.

FIGURE 13. CsCl and sucrose gradients of ^{32}P-labelled F pili complexed with ^{3}H-guanosine R17.

(A) A composite of three different CsCl density gradients. One (□-□) contained only ^{3}H-guanosine R17, one contained only ^{32}P-labelled F pili (o-o) while a third contained a previously incubated mixture of ^{32}P-F pili and ^{3}H-guanosine R17. The ^{32}P cpm are represented as closed squares (■-■), while the ^{3}H cpm are closed triangles (▲-▲).

(B) A composite of three different sucrose sedimentation velocity gradients containing the same samples as in (A). The symbols for both types of gradients are the same.


Density of CsCl Soln at 25° (g/cm3)(●—●)
CPM x 10-3
A
B
R17
R17+Pili
Pili
R17
R17+Pili
Pili
1.5
1.4
1.3
1.2
5
4
3
2
1
0
4
3
2
1
0
2
4
6
8
10
12
14
16
18
20
22
Fraction Number

The results are shown in Figure 13B. Only half of the ^{32}P counts derived from the ^{32}P-labelled F pili previously incubated with ^{3}H-R17 was found associated with a peak representing R17 - F pilus complexes. The other half of these counts was found in a peak co-banding with free F pili. No ^{32}P counts were found in any peaks other than those representing free F pili or F pili associated with R17 particles. This suggested that the ^{32}P was covalently linked to the F pilus molecule.

Further evidence from SDS-polyacrylamide gel electrophoresis of ^{32}P-labelled F pili supported this conclusion. A sample containing 2×10^4 cpm ^{3}H-aa-R17 (8×10^{11} p/sample) and 2×10^3 cpm ^{32}P-labelled F pili was run on a 12.5% polyacrylamide gel and stained with Coomassie blue as described in Chapter II. Other samples containing only ^{3}H-aa-R17 or ^{32}P-labelled F pili were also run on separate gels. The positions of the R17 coat protein and A protein as well as the F pilin protein were measured relative to the dye front which had travelled 6 cm down the gel (7.5 cm total length).

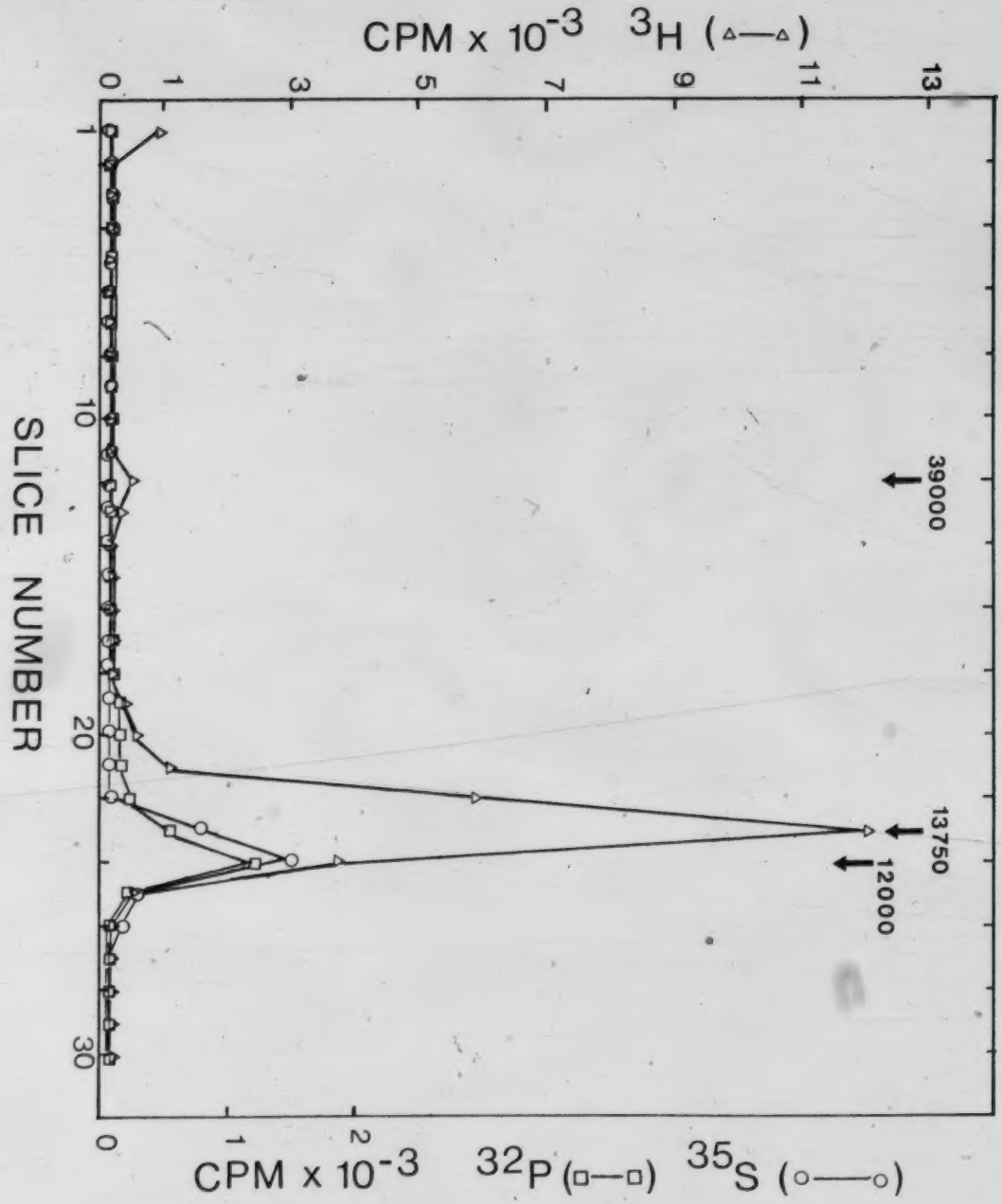
The gels were sliced into 2 mm sections and were incubated in 1 ml of 15% NH_4OH in covered vials at 37° for 24 hours. The NH_4OH was evaporated in a 60°C oven placed in a fume hood and the samples were counted in 5.0 ml Bray's scintillation fluid.

A graph relating ^{32}P or ^{3}H cpm to position in the gel is given in Figure 14. The ^{32}P radioactivity was found exclusively at a position in the gel consistent with a molecular weight of 12,000, the molecular weight of F pilin.

The ^{32}P-labelled F pili were used in the estimation of the number of phosphate groups per F pilin monomer. The specific activity of the ^{32}P in the F pilus preparation was calculated by determining the

FIGURE 14. SDS polyacrylamide gel electrophoresis of ^{32}P and ^{35}S-labelled F pili.

Gels containing 2 x 10^3 cpm of ^{32}P or ^{35}S-labelled F pili derived from *E. coli* ED2687 or 2 x 10^4 cpm of R17 labelled with a ^{3}H-amino acid mixture were sliced into 2 mm sections (for a total of 30 sections) and assayed for radioactivity as described in the text. The molecular weight of the radioactive species in the gels was estimated using standards as described in Figure 11.


CPM x 10-3 3H (△—△)
0
1
3
5
7
9
11
13
SLICE NUMBER
1
10
20
30
39000
13750
12000
CPM x 10-3 32P (□—□) 35S (○——○)
0
1
2

concentration of phosphate in the medium and relating this to the ^{32}P cpm for 1.0 ml of medium. This gave a specific activity of 10^{13} cpm/mole of phosphate. Using the relationship of 10^3 cpm/µg of ^{32}P-F pilin as estimated from the intensity of the band stained with Coomassie blue, there is approximately 2 - 2.5 phosphate groups per molecule of F pilin which is in accordance with values determined by other workers.

Radioactivity was used to determine whether phosphate-containing contaminants could be effectively separated from F pili using the purification procedure described for ^{32}P-labelled F pili. In this experiment a one-litre culture of HB11 F^- cells grown in glucose CTMM + aa + 5 mCi of ^{33}P ($H_3{}^{33}PO_4$ in water neutralized with 0.1 N NaOH) was mixed with a one-litre culture of ED2687 containing 5 mCi of ^{32}P. The F pili were purified using the 30% sucrose method to remove the F pili from the cells. After the cells were removed by centrifugation and the sucrose dialyzed away, the ^{32}P- and ^{33}P-labelled material was pelleted and banded on a CsCl density gradient. A 50 µl portion of each fraction from the CsCl gradient was assayed for ^{32}P and ^{33}P radioactivity as well as for R17 phage attachment using 10^{12} ^3H-G-R17 particles/sample. As may be seen in Figure 15, all of the ^{33}P radioactivity derived from the F^- culture was found at the bottom of the gradient with contaminating cells. Assuming that this method of purification could give rise to as much cellular debris and membrane contaminants in the F^- culture as the F^+ culture, it can then be concluded that very few phosphate-containing contaminants are present in F pili preparations. The vesicle-like material found in some F pili preparations probably does not contain cell-associated phosphate.

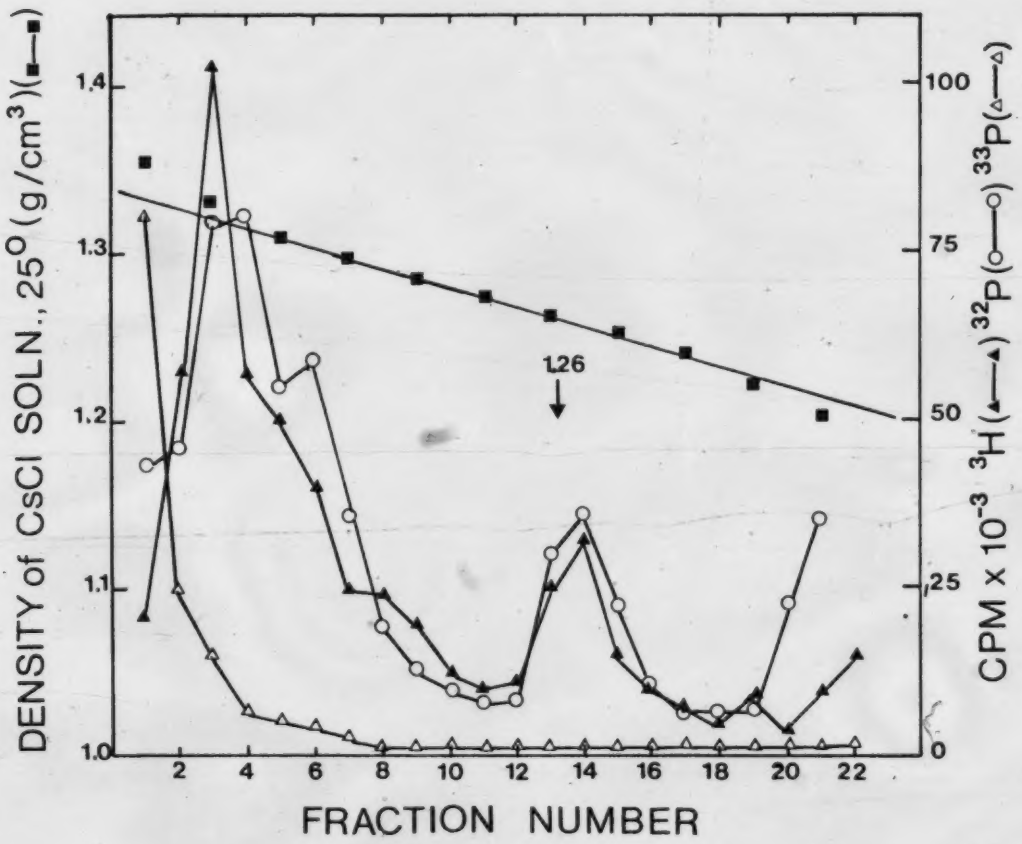


FIGURE 15. Separation of F pili preparations from F^- cellular debris.

A CsCl density gradient of F pili prepared from a liquid culture of ^{32}P-labelled ED2687 cells mixed with an equal volume of HB11 F^- cells labelled with ^{33}P was performed as described in the text. Each fraction was assayed for ^{33}P (Δ-Δ) and ^{32}P (o-o) radioactivity as well as ^{3}H-guanosine R17 phage attachment using the filtration assay (▲-▲).

It should also be noted that F pili can be easily labelled with ^{35}S-methionine (New England Nuclear Corp.). A 100 ml volume of glucose CTMM-aa was inoculated with ED2687 and 1.25 mCi ^{35}S-methionine (0.1 mM) was added at the time of inoculation. The ^{35}S-F pili were purified as described previously and banded on a CsCl isopycnic density gradient (Figure 12B). Two sharp peaks at $\rho^{5°}$ = 1.26 and 1.30 g/cm^3 were obtained and the presence of F pili was checked by electron microscopy. This method gave ^{35}S-labelled pili with a specific activity of 500 cpm/µg of F pili. This labelled F pili was used to confirm the position of the ^{32}P-labelled F pili in SDS-polyacrylamide gels (see Figure 14).

C. CONCLUSION

In 1971, Brinton described in a review article the amino acid composition, carbohydrate and phosphate content of F pilin. Except for the protocol for his purification procedure for F pili published five years later (Minkley et al., 1976), no methodology for this data has been reported to date. In 1972, Beard, Howe and Richmond published a different purification procedure for F-type R pili but insufficient amounts of pure F pili material discouraged them from proceeding with a detailed chemical analysis.

Brinton's findings, reported in 1971, have very recently been corroborated by Date, Inuzuka and Tomoeda (1978). Thus it has taken seven years for the preliminary chemical and physical characteristics of F pili to be established. Brinton has not continued with the chemical analysis of F pili and whether Date et al. will continue is yet to be seen.

The results of our investigations which were completed in 1974

confirmed Brinton's findings in 1971. Namely, F pili had one principal subunit, F pilin, of molecular weight 11,900, which contained small amounts of carbohydrate and phosphate. The isoelectric point of F pili was pH 3.9 to 4.0. F pili band in CsCl at $\rho^{5°}$ = 1.26 and to a lesser extent at $\rho^{5°}$ = 1.30 depending on the plasmid the bacterial strain is harboring. F type R pili band at $\rho^{5°}$ = 1.31 g/cm^3.

In 1974 three options were available to us to achieve a more detailed chemical analysis of F pili. (1) The methodology and bacterial strains in use at that time could be used to stockpile sufficient quantities of pure F pili. (2) Microtechniques in analysis and protein sequencing could be used to characterize the small amounts of F pili obtainable with short-term stock-piling. (3) A search for a bacterial strain which produced pili in large quantities could be undertaken.

At about this time, D.E. Bradley (1974b) reported on a highly multipiliated strain of P. aeruginosa K, which he kindly donated to us. With this strain, pili could be purified in large quantities to almost 100% purity. Moreover, the wild type strain, K, produced sufficient pili to warrant a comparison of physical and chemical properties between the two strains. It was therefore evident that with Pseudomonas pili it would be possible to undertake the complete sequencing of the pilus subunit with conventional sequencing techniques. Since an ultimate objective was the comparative analysis of amino acid sequence data from a variety of pilus types, the time seemed opportune to begin working on P. aeruginosa pili while accumulating preparations of F pili for eventual sequencing studies. The remainder of this thesis is therefore concerned with the purification and partial characterization of pili isolated from P. aeruginosa K.

CHAPTER V

COMPOSITION AND MOLECULAR WEIGHT OF PILIN PURIFIED FROM PSEUDOMONAS AERUGINOSA K

A. INTRODUCTION

Although the genetic origin of PSA pili is unknown (see Chapter I) and there is no evidence that they are involved in conjugational processes, they bear certain similarities to conjugative pili such as the F pili of E. coli as discussed in Chapters III and IV. For example, they act as receptors for a number of bacteriophages including both RNA and DNA-containing bacteriophages and bacteriophages with long non-contractile tails (Bradley, 1977a). Since both polar pili and F pili apparently mediate phage infection through some type of pilus retraction mechanism (Bradley, 1972; Novotny and Fives-Taylor, 1974; Paranchych, 1975), it is of considerable interest to compare the physical and biochemical properties of these two pilus types.

As a first step in this direction, a study of P. aeruginosa K (PAK) PSA pili was undertaken. This strain, which promotes infection by at least six bacteriophages, was used by Bradley (1977a) to isolate a mutant which was resistant to these pilus-dependent bacteriophages. This mutant (PAK/2PfS) (Bradley, 1974b) was multi-piliated and was apparently resistant to bacteriophages by virtue of its inability to retract its pili. While the wild-type strain, PAK, was a relatively good producer of pili in its own right, the multi-piliated strain, PAK/2PfS, probably contained at least 10-fold more pili than PAK.

The present investigation was carried out to establish the structural relatedness of PAK and PAK/2PfS pili and to determine their

biochemical composition. A purification procedure for PAK and PAK/2PfS pili which attains greater than 99% purity is described here as well as a number of observations relating to their physical and chemical properties.

B. RESULTS AND DISCUSSION

1. Choice of producer strain

As shown in the electron micrographs in Figure 16, the wild-type *P. aeruginosa* strain K is itself a reasonably good producer of polar pili, while the mutant (PAK/2PfS) is extremely heavily piliated. PAK cells apparently do not produce the equivalent of the type 1 pili seen on *E. coli* (Brinton, 1965), although numerous flagella are produced.

2. Pili purification procedure

PAK or PAK/2PfS bacteria were grown on solid TSB in shallow pans (27 cm x 38 cm x 1.25 cm), and were harvested by scraping the surface of the agar and suspending the cells from 36 trays (~160 grams wet weight) in 500 ml 15% sucrose in SSC buffer pH 7.0. The cells were then stirred with a magnetic stirrer at 5° for a minimum of 24 hours, after which they were passed through a sieve to remove the bits of agar, and then blended for 2 min in the cold in 200 ml portions at 2000 rpm with a Sorvall Omnimixer. The cells were removed from the solution by centrifugation at 10,000 x g for 15 min, after which the supernatant solutions was dialyzed for 72 hours in 2-foot lengths of dialysis tubing against constantly running tap water at 15° to 17° in a cold room. Ammonium sulfate was then added to the dialysate to 50% saturation, and the solution was allowed to stand overnight at 5° during which time

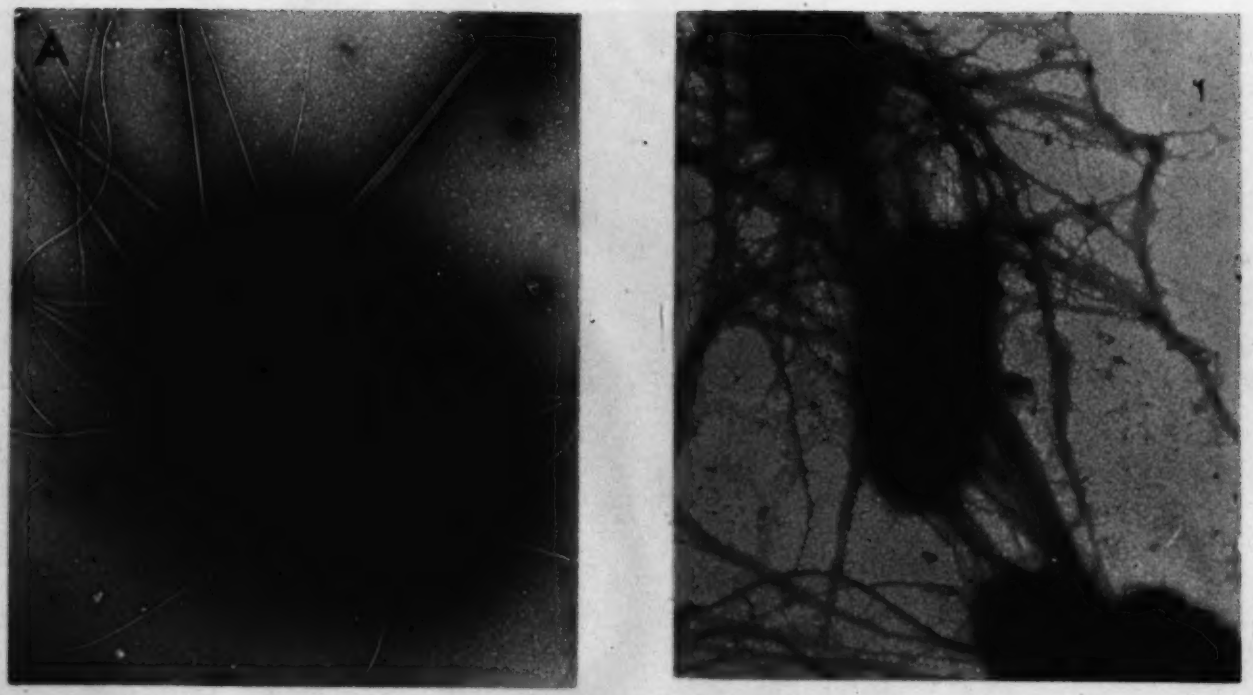


FIGURE 16. Electron micrographs of strains Pseudomonas aeruginosa K (PAK) and K/2PfS.

This plate illustrates the large numbers of pili per cell on PAK (A) and PAK/2PfS (B). The parent strain, PAK, produces more flagella than the multi-piliated mutant PAK/2PfS. The specimens were over-stained to achieve clearer visualization of the pili. Magnification: A, X11,000; B, X7500.

the protein precipitate floated to the top. Most of the clear portion of the solution was removed by syphoning, while the precipitate was pelleted by centrifuging for 1 hour at 27,000 x g in a Sorvall Centrifuge. The precipitate was dissolved in approximately 200 ml of SSC buffer and was then clarified by centrifugation at 3,000 x g for 10 min. It was important at this stage to insure that no pili were sedimented along with the cellular debris. If a white opalescent pellet formed, the pellet and supernatant solutions were recombined and diluted with 50 ml portions of SSC buffer until only cellular debris was pelleted in the low speed centrifugation step.

To remove flagella, the principal contaminant of the pili preparation, the supernatant solution containing 1 to 2 A_{280} units per ml, was brought to approximately 20% saturation with ammonium sulfate (10% w/v) and kept at 5° for 2 hours. The pili precipitated out, while the flagella remained in solution. A 20% saturated solution of ammonium sulfate removes pili readily at this stage of the purification procedure but a 50% saturated solution is required to precipitate pili out of the crude culture medium. The reasons for this are not entirely clear but may be related to the fact that the pili at this stage are usually in large aggregates whereas they are in a more dissociated state and less concentrated in the growth medium. Since the ratio of PAK/2PfS pili to flagella was much greater than that of PAK pili to flagella, PAK/2PfS pili preparations required only one cycle of removal of flagella, while PAK required two cycles of ammonium sulfate precipitation (10% w/v).

The pili were redissolved in SSC buffer at a concentration of approximately 1 mg/ml and 15 ml was placed on a 20 ml discontinuous

sucrose gradient consisting of 5 ml portions of 40, 50, 60 and 70% sucrose in SSC buffer, pH 7.0. The gradients were centrifuged for 20 hours at 5° using a SW 27.1 rotor at 20,000 rpm. The cellulose nitrate tube was punctured with a 20 gauge needle just below the prominent band of pili material. After removal from the gradient, the pili suspension was dialyzed against SSC buffer to remove sucrose and was then rebanded in CsCl as described in Materials and Methods. The pili band was again retrieved with a syringe, dialyzed against SSC buffer to remove CsCl and then stored in a frozen state at -20°. This procedure yields about 10 mg purified PAK/2PfS pili per 100 g (wet weight) of cells and about one tenth of this amount of pili using the wild-type strain, PAK.

When salt-free pili were required for certain chemical studies, the pili were pelleted by centrifuging for 1 hour at 27,000 x g and redissolved in distilled deionized water. After two cycles of centrifugation, the pili suspension was further dialyzed for 24 hours against deionized water and then lyophilized. The dried powder was stored at -20°.

3. Criteria of purity

The purity of the pili at various stages of the purification procedure was monitored by means of electron microscopy and SDS disc gel electrophoresis. Figure 17 shows electron micrographs of a PAK/-2PfS pili preparation before and after the removal of flagella with ammonium sulfate treatment, while Figure 18 shows typical gel patterns before and after the removal of flagella and after subjecting the pili preparation to CsCl density gradient centrifugation. It is evident from Figures 17 and 18 that a high degree of pili purity was achieved with the foregoing purification procedure. When polyacrylamide gels

FIGURE 17. Electron micrographs of PAK/2PfS pili during the purification procedure.

The detailed purification procedure is given in the text. A crude pili concentrate (A) containing contaminating flagella was treated with 10% (w/v) ammonium sulfate which precipitated the pili and left the flagella in solution. The resultant pili preparation (B) contained no flagella. Magnification: A, X17,500; B, X11,000.

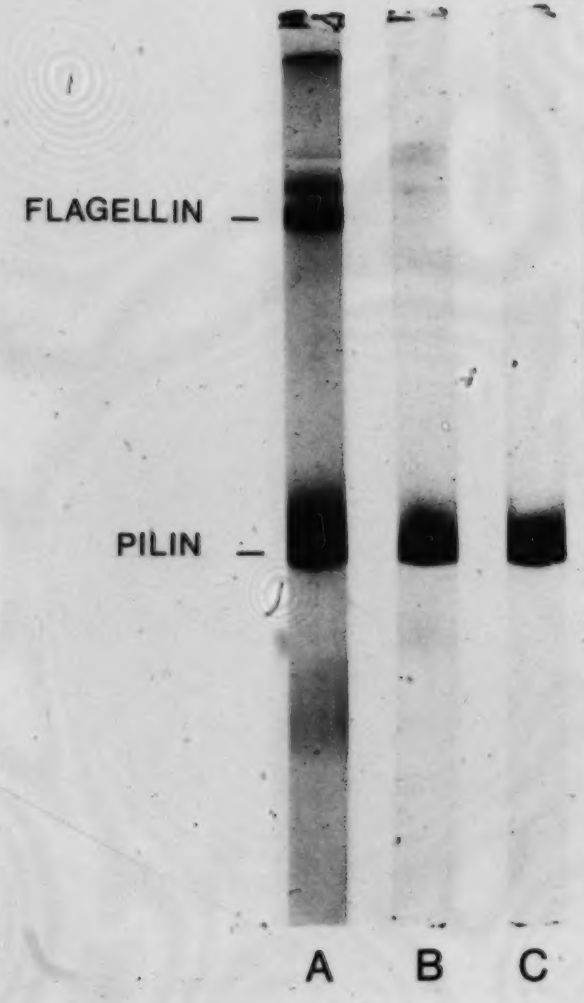


FIGURE 18. SDS-polyacrylamide disc gel electrophoresis of PAK/2PfS pili during the purification procedure.

A detailed account of the purification procedure appears in the text. The gels, containing 12.5% acrylamide and 0.1% SDS, represent samples taken from a preparation of PAK/2PfS pili at steps before (A) and after (B) 10% (w/v) ammonium sulfate precipitation to separate pili from flagella and after CsCl isopycnic centrifugation (C) to separate pili from large molecular weight contaminants. The amount of protein in each gel is 20 - 50 µg as estimated by the Lowry procedure. The gel buffer and tank buffer was 0.05 M sodium phosphate buffer, pH 7.2, 0.1% SDS. The gels were run at 5 ma/gel for 90 min and were stained with Coomassie blue.

were overloaded with pilin (30 µg), a second, faint band with a relative mobility corresponding to a molecular weight of 32,000 consistently appeared above the pilin band. To determine whether this material represented a minor protein component of pili or a dimer of pilin, approximately 100 µg was extracted from each of 10 preparative SDS-polyacrylamide gels as described in the preparation of pilin as an antigen. (see Chapter II). The eluted protein was treated with 0.05 N acetic acid in acetone to remove SDS, then hydrolyzed in 6 N HCl *in vacuo* and subjected to an amino acid compositional analysis using a Durrum D500 amino acid analyser. The analysis showed that the 32,000 molecular weight component had the same amino acid composition as pilin (data not shown). It was therefore concluded that the 32,000 molecular weight band was a dimer of pilin.

It is to be noted that the amino acid composition analysis yielded nearly integral values for the moles of amino acid/mole of pilin. Moreover, the N-terminal amino acid analysis showed only one amino acid residue per mole of protein (see Chapter VI). These observations provide an additional indication of the high level of purity of the pili preparations. Purified pili solutions (suspended in water) were found to have an absorbance at 280 nm of 0.78 ml/mg. Protein estimations were done using the Lowry procedure (Lowry *et al.*, 1951).

4. Buoyant density in cesium chloride and sucrose

To determine the buoyant density of pili in CsCl, gradients containing either PAK or PAK/2PfS pili were run as described in Materials and Methods. The refractive index readings were converted to density values at 5°. SSC buffer (1.0 ml) was added to each fraction and the absorbance at 280 nm was determined in a Beckman DBG spectrophoto-

meter. Figure 19 shows that the density in CsCl of pili from both PAK and PAK/2PfS strains was 1.295 g/cm^3.

The buoyant density of PAK or PAK/2PfS pili in a sucrose gradient was determined as described in Materials and Methods. The absorbance at 280 nm was recorded on every other fraction and the resulting distribution of absorbance versus density is shown in Figure 20 . It may be seen that the density in sucrose of both PAK and PAK/2PfS pili was 1.221.

5. Isoelectric focusing

Isoelectric focusing of purified pili material was performed as described in Materials and Methods. The results of the experiment are shown in Figure 21 where it may be seen that the pI of intact PAK or PAK/2PfS pili was found to be 3.9. It is to be noted that problems of pili precipitation were encountered if ampholytes of a pH range narrower than 3 - 10 were employed. The pI of the pili remained unchanged if the polarity of the electrodes was reversed, indicating that precipitation effects were negligible for the conditions used.

6. Molecular weight determination of pilin subunits

The molecular weight of pilin subunits was estimated using SDS gel electrophoresis as described by Weber and Osborn (1969). As mentioned above, a small amount of pilin was always found in dimer form, even after prolonged heating with higher concentrations of SDS. A comparison of the relative mobility of pilin with that of a number of proteins of known molecular weight is shown in Figure 22 . It may be seen that a plot of the relative mobilities of the various proteins versus the log of the molecular weights yielded a molecular weight value for PAK/2PfS pilin of 17,800 ± 300. The same molecular weight value was

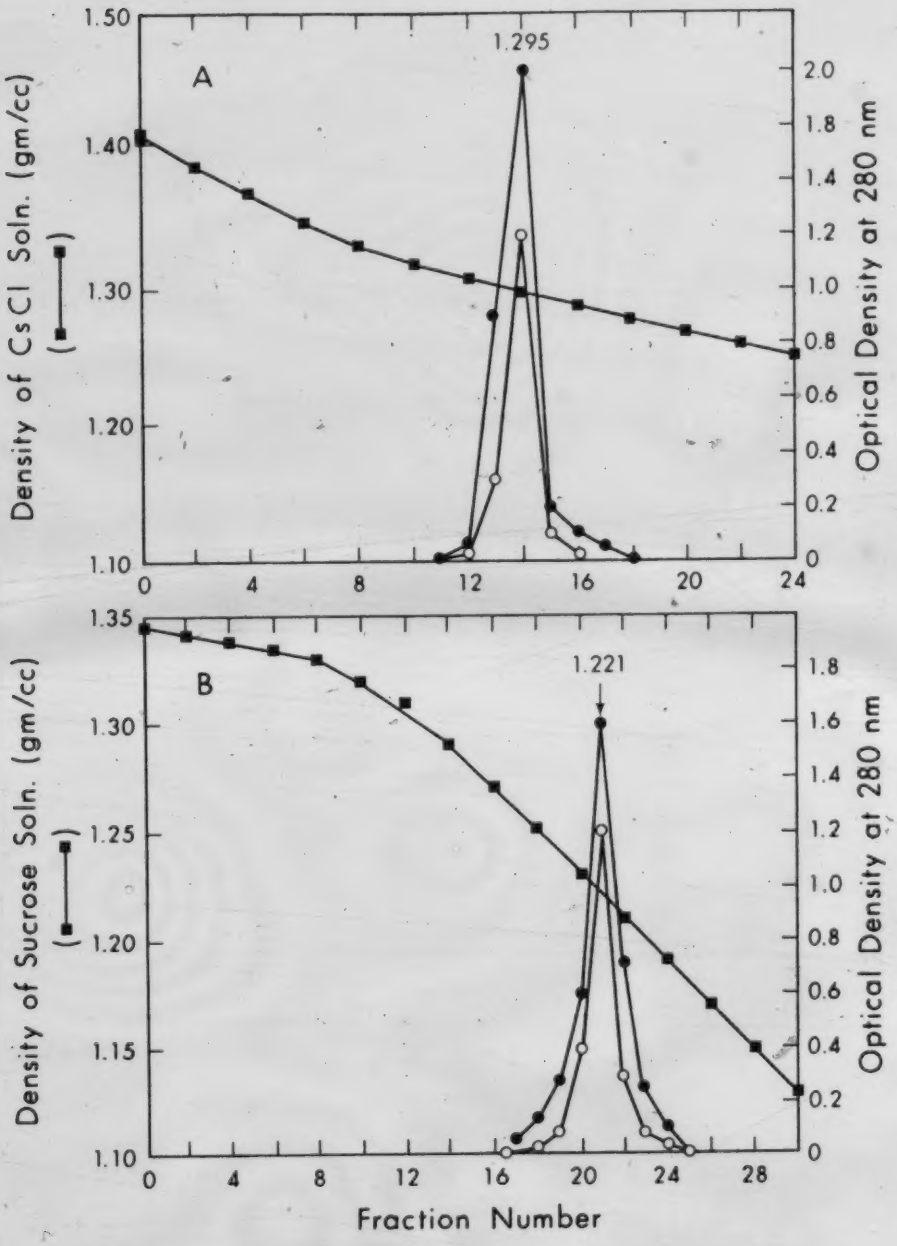
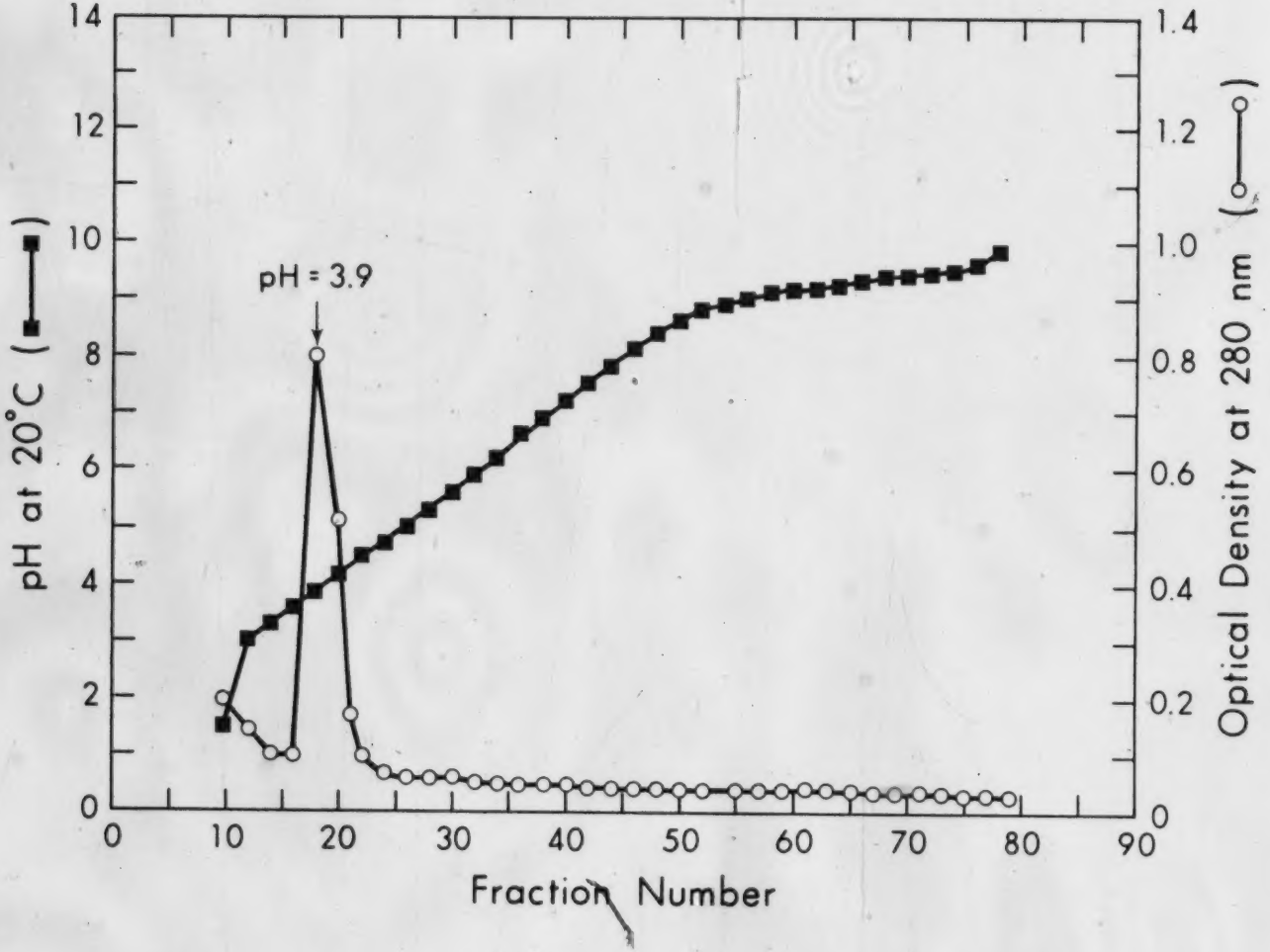


FIGURE 19,20. Isopycnic centrifugation of purified PAK and PAK/2Pfs pili in CsCl and sucrose density gradients.

19 Absorbance at 280 nm of PAK and PAK/2PfS pili in CsCl gradient.
20 Absorbance at 280 nm of PAK and PAK/2PfS pili in sucrose gradient. (■-■), density at 5°. (o-o), absorbance of PAK pili. (●-●), absorbance of PAK/2PfS pili.

FIGURE 21. Isoelectric focusing of purified PAK/2PfS pili.

Isoelectric focusing of 1.0 mg of purified PAK/2PfS pili was performed using an ampholyte solution pH range 3 - 10 in a 110 ml linear sucrose gradient for 40 hours at an average voltage of 400 V. (■-■), pH; (o-o), absorbance at 280 nm.


pH = 3.9
pH at 20°C (■—■)
Optical Density at 280 nm (○—○)
Fraction Number
0
2
4
6
8
10
12
14
0.2
0.4
0.6
0.8
1.0
1.2
1.4
10
20
30
40
50
60
70
80
90

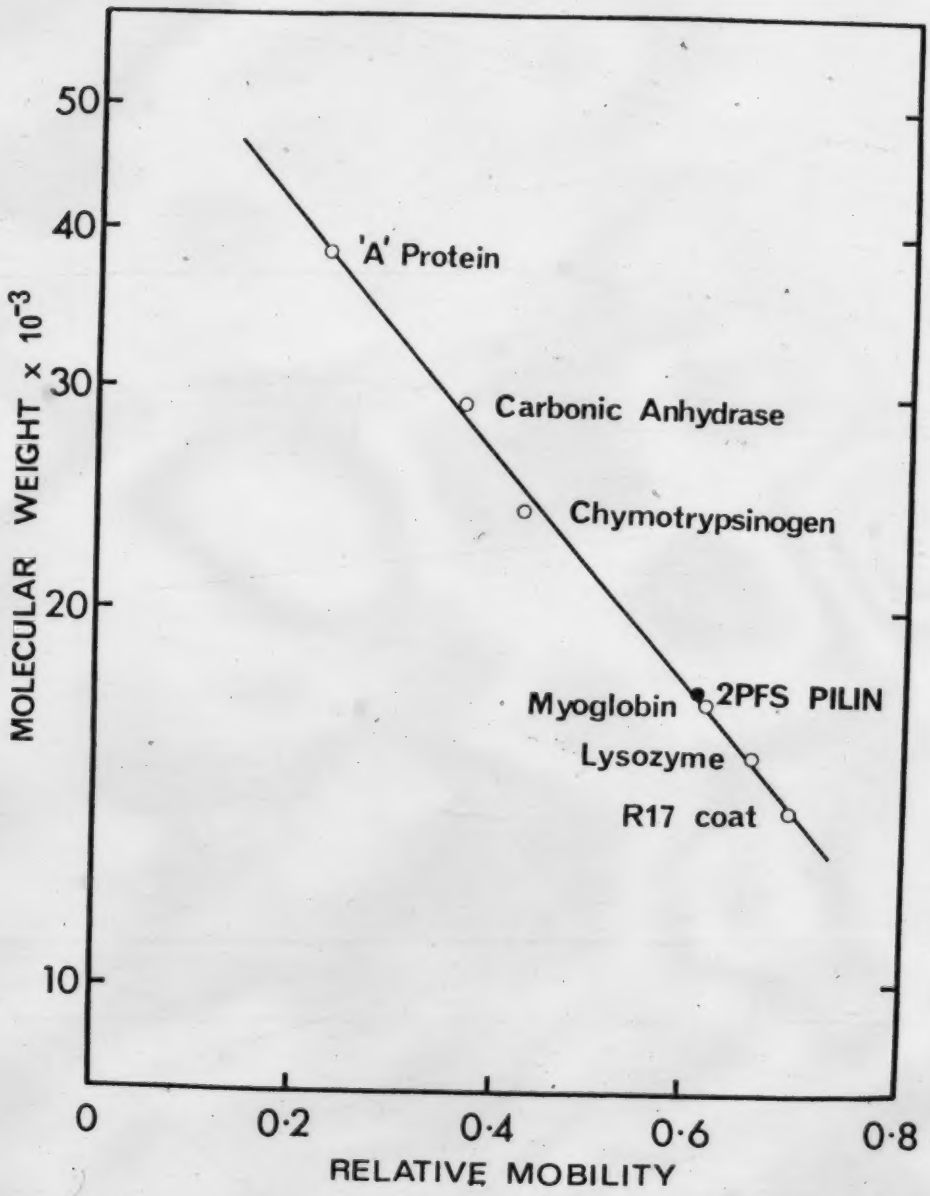


FIGURE 22 . Determination of the molecular weight of PAK/2PfS pilin by SDS polyacrylamide gel electrophoresis.

The molecular weight of the protein standards was taken as stated by Weber and Osborn (1969). The relative mobility was calculated by comparing the migration (cm) of the protein to migration (cm) of bromphenol blue.

also obtained for PAK pilin (not shown). The molecular weight value obtained for PAK pilin is much larger than the molecular weight value of 11,400 reported for F pilin (Minkley et al., 1976; or 11,800, Date et al., 1978), but agrees well with the value of 18,400 for PAK pilin obtained on the basis of amino acid composition data (see Table 6). This molecular weight agrees closely with that of pilin from N. gonorrhoeae (18,300, Hermodson et al., 1978; 18,000 - 19,500, Robertson et al., 1977), pilin from Moraxella nonliquefaciens (17,000, Froholm and Sletten, 1977) and type 1 pilin from E. coli (Brinton, 1965), which were all determined from amino acid compositional data.

7. Amino acid composition of PAK pilin

The procedure used for subjecting PAK pili to acid hydrolysis in vacuo is described in Materials and Methods. The amino acid compositional values reported are averaged values for 28, 48 and 72 hour hydrolysis periods. Serine and threonine were estimated by extrapolating to zero time. Tryptophan was estimated by hydrolyzing the pilin sample in p-toluene sulfonic acid and 0.2% 3-(2-aminoethyl) indole (kindly supplied by L.B. Smillie) according to the method of Liu and Chang (1971). Spectrophotometric determination of tryptophan (Goodwin and Morton, 1946) confirmed the value of two tryptophan residues per pilin subunit. Oxidizing the PAK pilin with performic acid according to the method of Hirs (1967b) gave an estimate of cysteine and methionine residues as cysteic acid and methionine sulfone. Cleavage with cyanogen bromide (see Chapter VI) gave the number of methionine residues as homoserine. The number of methionine residues increased to 3 when treated with these agents. The amino acid composition of PAK and PAK/2PfS pilin is given in Table 5. The PAK pilin analyses usually contained 0.1 - 0.2 histidine residues which were disregarded. The molecule

TABLE 5

Amino Acid Compositions of PAK and PAK/2PfS Pilin

Amino Acid	Residues per 17,800 daltons	
	PAK	PAK/2PfS
Lys	14.7	14.8
His	0	0
Arg	3.4	4.0
Asx	14.9	14.9
Thr	14.7	15.0
Ser	10.0	9.9
Glx	14.7	14.9
Pro	9.4	9.7
Gly	18.2	17.7
Ala	24.1	23.8
1/2 Cys	N.D.	3.9
Val	9.2	9.0
Met	N.D.	2.1
Ile	11.8	11.7
Leu	14.4	14.0
Tyr	2.3	2.2
Phe	2.3	2.3
Trp	1.9	2.0

The samples were hydrolyzed *in vacuo* at 110° in 6 N HCl containing 0.1% phenol for 24, 48 and 72 h. The analyses are normalized to a total of 173 residues.

contains a relatively large number of proline residues when compared to F pilin which contains none.

Recently Date et al. (1978) analyzed F pilin and found the amino acid composition was similar to that reported by Brinton (1971). Furthermore circular dichroism studies suggested that nearly 70% of F pilin was helical in structure. This is probably not the case with PAK pilin, however, since the latter contains many more proline residues. As shown in Table 6, PAK and type 1 pilin contain a relatively large proportion of non-polar amino acids, i.e., about 43%, but the proportion of non-polar amino acids in F pilin is higher still at 53%. This may account for our experience that PAK pili are much easier to dissociate from each other and from cell debris than F pili. Consequently, PAK pili are considerably easier to purify than F pili. This is also apparently true for type 1 pili (Brinton, 1965) and pili of N. gonorrhoeae (Robertson et al., 1977; Hermodson et al., 1978).

8. Immunological relatedness of PAK and PAK/2PfS pili

It is evident from the amino acid composition data in Table 5 that the pilin subunits of PAK and PAK/2PfS pili are probably identical or closely related polypeptides. However, the possibility existed that PAK and PAK/2PfS pili might be antigenically dissimilar. The immunological relatedness of these two types of pili was therefore examined by performing microimmunodiffusion analyses on the purified pili and the corresponding antisera. The results of the experiment are shown in Figure 23.

It is to be noted that although the antisera were prepared by challenging rabbits with pilin preparations suspended in pulverised SDS-polyacrylamide gel material (see Materials and Methods), similar

TABLE 6

Polar and Nonpolar Amino Acid Residues
of Several Types of Pilin

Amino Acid Character	Per Cent			
	PAK pilin[1] (174)[5]	F pilin[2] (124)[5]	Type 1 pilin[3] (163)[5]	*N. gonorrhoeae* pilin[4] (208)[5]
Acidic (Asp, Glu)	17.3	9.7	20.3	13
Basic (Lys, His, Arg)	11.0	8.0	4.9	25
Uncharged Polar (Ser, Thr, Tyr, Gly, Cys)	28.3	29.0	31.3	26
Nonpolar (Ala, Leu, Ile, Val, Pro, Phe, Trp, Met)	43.4	53.2	43.6	36

[1]Calculated from the data shown in Table 5.

[2]Calculated from data reported by Brinton (1971).

[3]Calculated from data reported by Brinton (1965).

[4]Calculated from data reported by Robertson *et al.* (1977).

[5]Total amino acid residues per pilin subunit.

precipitation bands were formed with either intact or highly denatured pilin. Moreover, the specificity of the antiserum towards PAK/2PfS pili is shown by the absence of any reaction with purified F-type pili (Figure 23A). Figure 23B shows reactions of identity between anti-PAK antiserum and purified PAK and PAK/2PfS pilin. Similar results were obtained when anti-PAK/2PfS antiserum was used (Figure 23C). It was concluded that PAK and PAK/2PfS pili are antigenically similar and that the multi-piliated state of the PAK/2PfS strain is due to the overproduction of PAK polar pili rather than the formation of a new type of surface structure.

9. Phosphate analysis

Since Brinton (1971) has reported that F pili contain two phosphate residues and one residue of D-glucose covalently linked to each pilin subunit; phosphate and sugar analyses were performed on PAK and PAK/2PfS pilin.

To assay for total inorganic phosphate, separate 1 - 2 mg samples of pure pili were hydrolyzed in acid and base as described in Materials and Methods, neutralized and subjected to the Fiske and Subba-Row (1925) method of phosphate estimation. Similar samples were ashed in sulfuric and perchloric acid and assayed for phosphorus using the microdetermination method of Chen et al. (1956). No phosphorus was found under any of these conditions where one molecule of phosphate per pilin subunit would have been detectable according to the sensitivities of the methods employed.

Similarly, no phosphate was detected in association with pilin bands in SDS-polyacrylamide gels. Pure pilin was subjected to SDS–polyacrylamide gel electrophoresis as previously described, then stain-

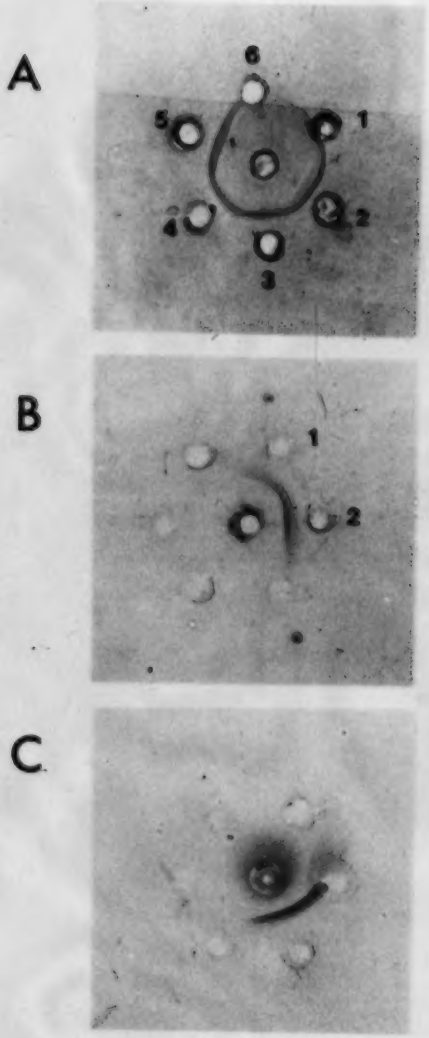


FIGURE 23. Gel diffusion pattern of anti-PAK and anti-PAK/2PfS pili serum versus PAK and PAK/2PfS pili.

The antisera and microimmunodiffusion plates were prepared as described in Chapter II.

A. The center well contained 2 µl of undiluted anti-PAK/2PfS pilin serum. The outer wells contained: (1) 10^7 PAK/2PfS cells; (2) 10 µg of intact PAK/2PfS pili; (3) 10 µg of PAK/2PfS pili in 0.1% SDS; (4) 10 µg of PAK/2PfS pili in 10 M urea; (5) 10 µg of PAK/2PfS pili in 0.1 N NaOH; (6) 10 µg of purified, intact F pili prepared from E. coli HB11.

B. The center well contained 2 µl of undiluted anti-PAK pilin serum. The outer wells contained: (1) 10 µg of PAK pili in 0.1% SDS; (2) 10 µg of PAK/2PfS pili in 0.1% SDS.

C. The center well contained 2 µl of undiluted anti-PAK/2PfS pilin serum. The outer well contained 10 µg PAK pili in 0.1% SDS.

ed for phosphoprotein using methyl green as reported by Cutting and Roth (1973). Since this method is capable of detecting one nmole of phosphate, 20 µg of pilin would be expected to produce a positive stained band if pilin monomers contained 1 mole phosphate/mole protein. Although 50 µg of pilin were subjected to this procedure, no positive reaction for phosphate could be identified. It was concluded from the foregoing observations that PAK and PAK/2PfS pilin does not contain covalently bound phosphate residues.

10. Carbohydrate analysis

Separate 1 mg samples of purified pili were hydrolyzed in acid and in base as described in Materials and Methods, neutralized and dried at 110°. The residues were dissolved in 0.1 ml 50% ethanol and subjected to paper chromatography as described in Materials and Methods. A ratio of 1 glucose equivalent of carbohydrate/molecule of pilin should yield approximately 10 µg carbohydrate/mg pilin. This amount would easily be detected using the $AgNO_3$-NaOH or the anisaldehyde-H_2SO_4 spray systems employed on the chromatograms. No carbohydrate was detected in PAK or PAK/2PfS pili after acid or alkaline hydrolysis at protein concentrations which would have allowed the detection of one glucose residue per 10 pilin molecules.

Purified pili were also tested for carbohydrate using anthrone reagent (Ashwell, 1957), orcinol reagent (Ashwell, 1957) and the phenol-sulfuric acid assay for glycoproteins (Hirs, 1967). No carbohydrate was detected with any of these procedures at protein concentrations which would have given positive results at a level of one glucose equivalent per two molecules of pilin.

Finally, 50 µg-amounts of purified pilin were subjected to SDS-

polyacrylamide gel electrophoresis and then stained by the periodic acid-Schiff (PAS) reaction (Clark, 1964) for glycoprotein. No positively staining bands containing carbohydrate material were detected in the gels.

It was concluded from the foregoing studies that PAK and PAK/2PfS pili do not contain carbohydrate residues.

11. Lipid analysis

The possible existence of lipid material in PAK or PAK/2PfS pili was tested by staining SDS-polyacrylamide gels of purified pili (50 µg) with saturated oil red-O in 60% ethanol for 18 - 24 hours (Abodeely et al., 1971). The gels were destained in 50% ethanol and rehydrated. Both PAK and PAK/2PfS pili were found to be negative with respect to the oil red-O staining process, indicating that the P. aeruginosa pili are probably lipid-free.

The use of these specific stains for phosphoproteins, glycoproteins and lipoproteins was checked by using appropriate standards. The phosphoprotein α-casein was stained positively using the methyl green stain while albumin gave a white precipitate. Rabbit serum (1 µl) was used as a source of glycoproteins and lipoproteins and gave red bands in both instances. Albumin on the other hand failed to react with either stain.

Recently Robertson et al. (1977) have detected phosphate and glucose in pili of N. gonorrhoeae and Date et al. (1978) have found phosphate and carbohydrate in F pili of E. coli. Thus, type 1 pili are the only other pili analyzed to the author's knowledge that are free of carbohydrate and phosphate.

12. Conditions for dissociating PAK pili to pilin

A search for conditions which caused PAK pili to dissociate to the monomer, pilin, without resorting to SDS, gave evidence that PAK pili are similar to F pili of E. coli in their resistance to dissociating conditions (Tomoeda et al., 1975; Date et al., 1978). Conditions which caused the dissociation of flagella to flagellin (DePamphilis and Adler, 1971) gave varying results for PAK pili. Heating a suspension of pili (0.5 mg) at 55° for 10 min to 1 hour gave no discernible dissociation as determined by electron microscopy. Treating similar suspensions of pili with 0.05 N HCl or 0.05 N NaOH resulted in the production of small discs which were easily discernible in the electron microscope. Pili solutions adjusted to pH 1, 3, 5, 9, 11 and 13 showed a gradual dissociation from pili to these discs with the pili remaining intact at pH 5 - 9, partially dissociated at pH 3 and 11 and fully dissociated at pH 1 and 13. Tomoeda et al. (1975) reported that F pili, when treated with 0.05 N HCl, dissociated into filaments of 1/2 diameter of intact F pili. Recently, Date et al. (1978) reported that F pili are dissociated into vesicles by acid but are unaffected by equivalent concentrations of base.

PAK pili were not dissociated in 8 M urea or 6 M guanidine-HCl. However, 10 M urea and heat at 60° for 1 h produced shortened pili with many small discs when checked by electron microscopy. Samples run in a Spinco Model E analytical ultracentrifuge equipped with UV optics showed that treatment with 0.1% SDS produced one component with a sedimentation coefficient of 3 - 4 S. Treatment with 10 M urea or 6 M guanidine-HCl resulted in a small amount of 3 - 4 S material with a larger amount of material of higher S value. Treatment with acid or base gave 6 S mat-

erial in one distinct species. Intact pili had a sedimentation value of 23 S although the band was fairly diffuse.

These results were similar to those of Date et al. (1978) who found that F pili lost phage-attachment capability when exposed to heat and were depolymerized by 0.1% SDS and 30 mM Sarkosyl and exposure to acid at pH 1.0.

Since PAK pili also require SDS to affect complete depolymerization while remaining intact in the presence of denaturing agents such as guanidine-HCl or urea, it seems likely that hydrophobic bonds are important in subunit interactions which stabilize the filamentous structure of pili. This is not surprising considering the hydrophobic nature of pili themselves regarding amino acid composition and behavior in aqueous solutions.

C. CONCLUSION

This chapter describes the purification procedure developed for PAK and PAK/2PfS pili. The method involves growing the bacteria on a solid medium in large pans, suspending the cells in SSC buffer containing 15% (w/v) sucrose and blending the cells to shear off the pili. After the cells are removed by centrifugation and the sucrose dialyzed away, the pili are subjected to one or two cycles of $(NH_4)_2SO_4$ precipitation followed by centrifugation in sucrose and CsCl density gradients. This procedure yields about 10 mg of pure pili per 100 g (wet weight) of PAK/2PfS cells while PAK cells yield one tenth of this amount.

As far as can be determined, PAK and PAK/2PfS pili contain a single polypeptide subunit of molecular weight 17,800 by SDS polyacrylamide gel electrophoresis, and appear to be free from moieties such as phos-

phate, carbohydrate and lipid. Isopycnic centrifugation studies revealed that PAK and PAK/2PfS pili have the same buoyant density in sucrose (1.221 g/cm^3) and CsCl (1.295 g/cm^3) density gradients. Both types of pili have an isoelectric pH of 3.9 as determined by isoelectric focusing. Of particular interest were the findings that the amino acid compositions of PAK and PAK/2PfS are the same and that the two types of pili appear to be serologically identical.

On the basis of the foregoing, it was concluded that there are no chemical or physical differences between PAK and PAK/2PfS pili. The mutation responsible for producing the multi-piliated state in PAK/2PfS is probably located in a gene other than the structural gene for PAK pilin.

One possible explanation for this multi-piliation could be that the mutation affects the gene product that controls retraction. Thus the equilibrium that has been hypothesized to exist between pilus elongation and retraction (Fives-Taylor and Novotny, 1974) could be disturbed in favor of elongation since the mutation causes the loss of the retraction mechanism.

CHAPTER VI

PARTIAL CHEMICAL CHARACTERIZATION OF PAK/2PfS PILIN

A. INTRODUCTION

This chapter concerns the identification of the N-terminal residue, an unusual amino acid, N-methylphenylalanine, and the sequencing of the first twenty-two amino acids at the N-terminal of PAK/2PfS pilin by sequential Edman degradation. In addition, the first two amino acids at the C-terminal were identified by digestion with carboxypeptidase A. The protein was cleaved by cyanogen bromide into at least three fragments which were separated by column chromatography.

Only PAK/2PfS pili have been used in these studies since insufficient quantities of PAK pili material were available.

B. RESULTS AND DISCUSSION

1. Automated N-terminal sequence analysis

Automated Edman degradations were performed on tein with a Beckman Model 890B Sequencer, utilizing either the standard 1 M Quadrol buffer system of Edman and Begg (1967) or the 0.1 M Quadrol system of Brauer et al. (1975). All reagents used were "Sequanal" grade (Pierce Chemical Co.) and all solvents were "distilled in glass" grade (Burdick and Jackson Laboratories Inc., Muskegon, Michigan). Residues were identified by a combination of three methods. Portions of the sequencer products, the anilinothiazolinone derivatives, were converted to the corresponding phenylthiohydantoin (PTH) by exposure to 1 M HCl at 80°C for 10 min (Edman and Begg, 1967) to permit identification by gas-liquid chromatography on a 1.2 m x 2 mm column of 10%

SP-400 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA.) using a Beckman GC-45 gas chromatograph (Pisano and Bronzert, 1969). Thin layer chromatography of these PTH derivatives on silica gel plates (F254, Merck and Co., Inc., Rathaway, N.Y.) utilizing the solvent systems chloroform:ethanol, 98:2 (v/v) and chloroform:ethanol:methanol, 88.2:1.8:10 (v/v) was also employed (Bridgen et al., 1975). In addition, amino acid analysis by a Durrum D-500 analyzer following hydrolysis of the thiazolinone derivatives with HI ("Ultrapure" grade, Alfa Chemicals Ltd., Montreal, Quebec) at 127° for 20 h was used (Smithies et al., 1971).

The first twenty-two amino acid residues were quantitated (nmol) and identified as:

NH_2-X-Thr-Leu-Ileu-Glu-Leu-Met-Ileu-Val-Val-Ala-Ileu-
143 125 139 75 91 74 116 68 85 98 61

-Ileu-Gly-Ile-Leu-Ala-Ala-Ileu-Ala-Ileu-Pro....COOH
61 67 46 54 54 55 42 46 25 15

Further sequencing determination at the N-terminal gave poor resolution of the residues since the protein was slightly soluble in the organic solvents used leading to a critical loss of material.

The N-terminal amino acid residue, and the phenylthiohydantoin derivatives (PTH-X), could not be identified by using standard PTH-amino acid derivatives either by gas-liquid chromatography or thin layer chromatography. Attempts to regenerate and detect by amino acid analysis the amino acid, X, either by acidic hydrolysis (5.7 M HCl at 110° for 24 h or 57% HI at 127° for 20 h) or alkaline hydrolysis (0.2 M NaOH/0.1 M $Na_2S_2O_4$ at 127° for 4 h) failed.

2. Identification of the N-terminal residue

To obtain sufficient PTH-X for identification using spectroscopic

techniques (0.2 mg), a combined total of 35 mg (2 µmol) of pilin were subjected to one Edman degradative cycle.

Preliminary proton magnetic spectra (PMR) of PTH-X and several PTH-amino acids (PTH-phenylalanine, PTH-leucine) and the mass spectrum (MS) of PTH-X indicated that PTH-X was probably PTH-N-methylphenylalanine (results not shown).

N-methylphenylalanine was synthesized by methylation of tertiary butyloxycarbonyl-phenylalanine dicyclohexylamine salt with methyl iodide in tetrahydrofuran in the presence of sodium hydride for 24 h at 21° (McDermott and Benoiton, 1973), with subsequent removal of the carboxyl- and amino-protecting groups by treatment with aqueous citric acid pH 3.0 and 50% (v/v) trifluoroacetic acid/dichloromethane respectively. Reaction of N-methylphenylalanine (90 mg) with 5% (v/v) phenylisothiocyanate in pyridine (2.4 ml) at 55° for 1 h gave the phenylthiocarbamyl derivative, which after drying in vacuo was converted to the PTH derivative by reaction with 1 N HCl (1 ml) at 80° for 10 min, the crude product being obtained by extraction with ethyl acetate (3 x 1 ml). Recrystallization from diethyl ether afforded colorless crystals (m.p. 113°).

Since the synthesized PTH-N-methylphenylalanine co-eluted as a single peak on a gas liquid chromatograph with PTH-X (Figure 24) and co-chromatographed as a single spot on silica gel thin layer chromatograms (E. Merck, precoated) using two solvent systems: R_f 0.94 with 1,2-ethanedichloride:acetic acid, 30:7 (v/v) (Edman, 1970) and R_f 0.33 with chloroform:ethanol, 98:2 (v/v)(Bridgen et al., 1975), MS, PMR and ultraviolet absorption spectroscopy were performed to compare the two compounds further. The MS of the synthetic PTH-N-methylphenylalanine

FIGURE 24. Gas liquid chromatogram of PTH-N-methylphenylalanine from PAK pilin.

GLC of (A) PTH-X; 2% of the product from the first Edman degradation of 5 mg (280 nmol) pilin and (B) PTH-N-methylphenylalanine together with a number of standard PTH-amino acids (5 nmol of each). Separations were made on a Beckman GC-45 gas chromatograph fitted with a 1.2 m x 2 mm i.d. column of 10% SP-400 on 100 - 120 mesh Supelcoport (Supelco Inc.) using helium carrier gas at 40 ml min^{-1}.

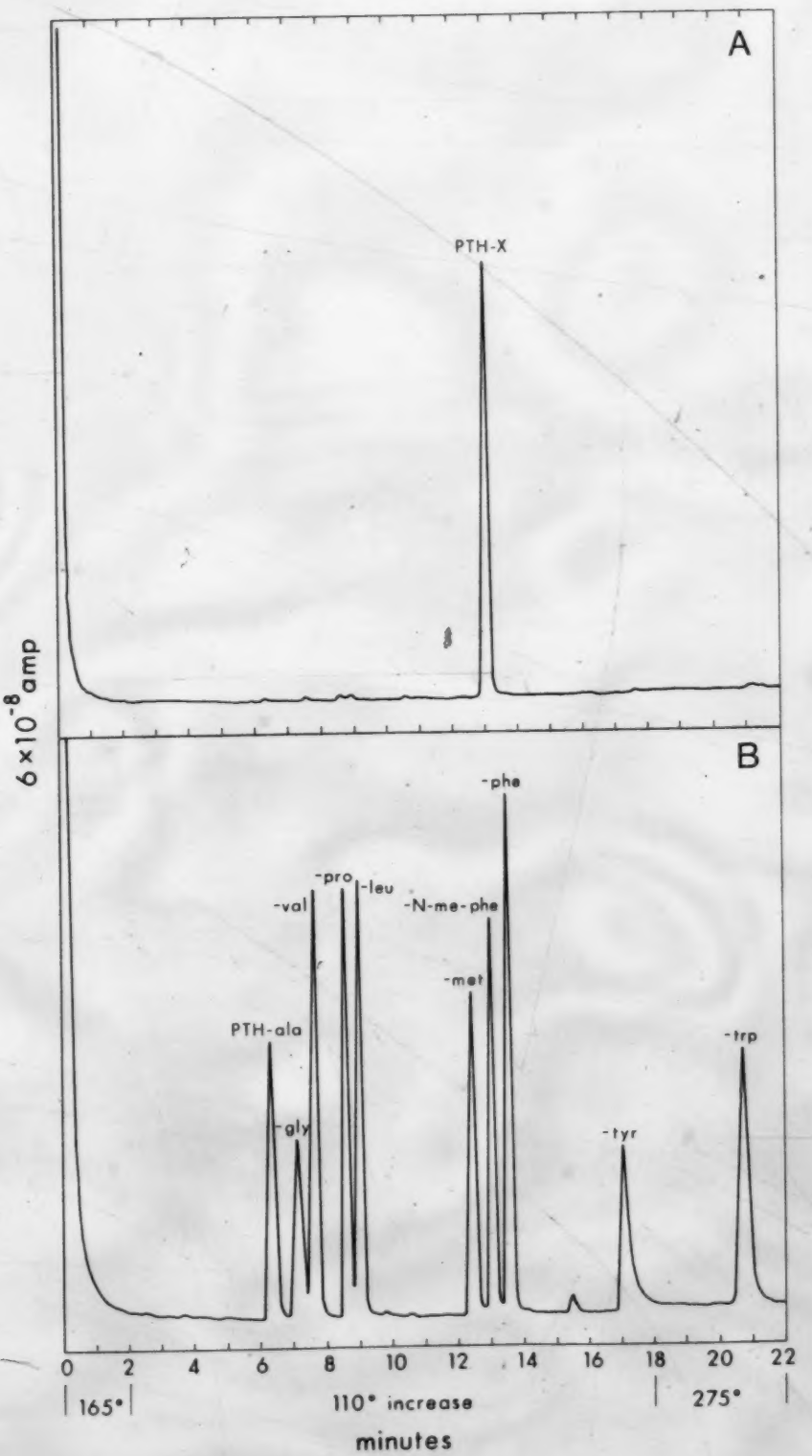

A
PTH-X
B
-phe
-pro
-leu
-val
-N-me-phe
-met
PTH-ala
-trp
-gly
-tyr
6×10⁻⁸amp
0
2
4
6
8
10
12
14
16
18
20
22
165°
110° increase
275°
minutes

FIGURE 25. MS of PTH-X and PTH-N-methylphenylalanine.

Both spectra were obtained on 10 µg or less of sample by direct probe, 70 eV electron impact ionization at an initial ion source temperature of 150°, using an AEI MS50 high resolution mass spectrometer in conjunction with a DS50 computer.

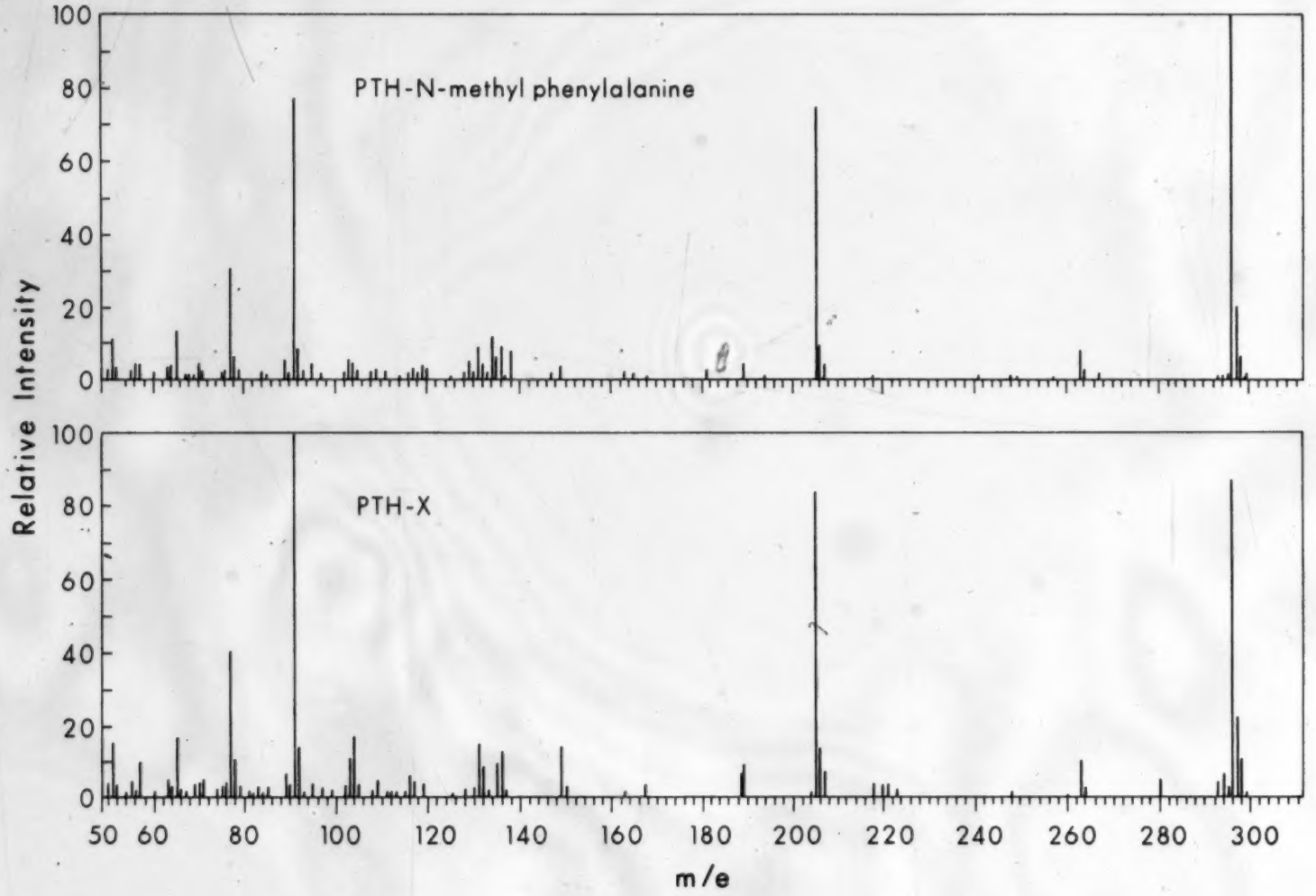
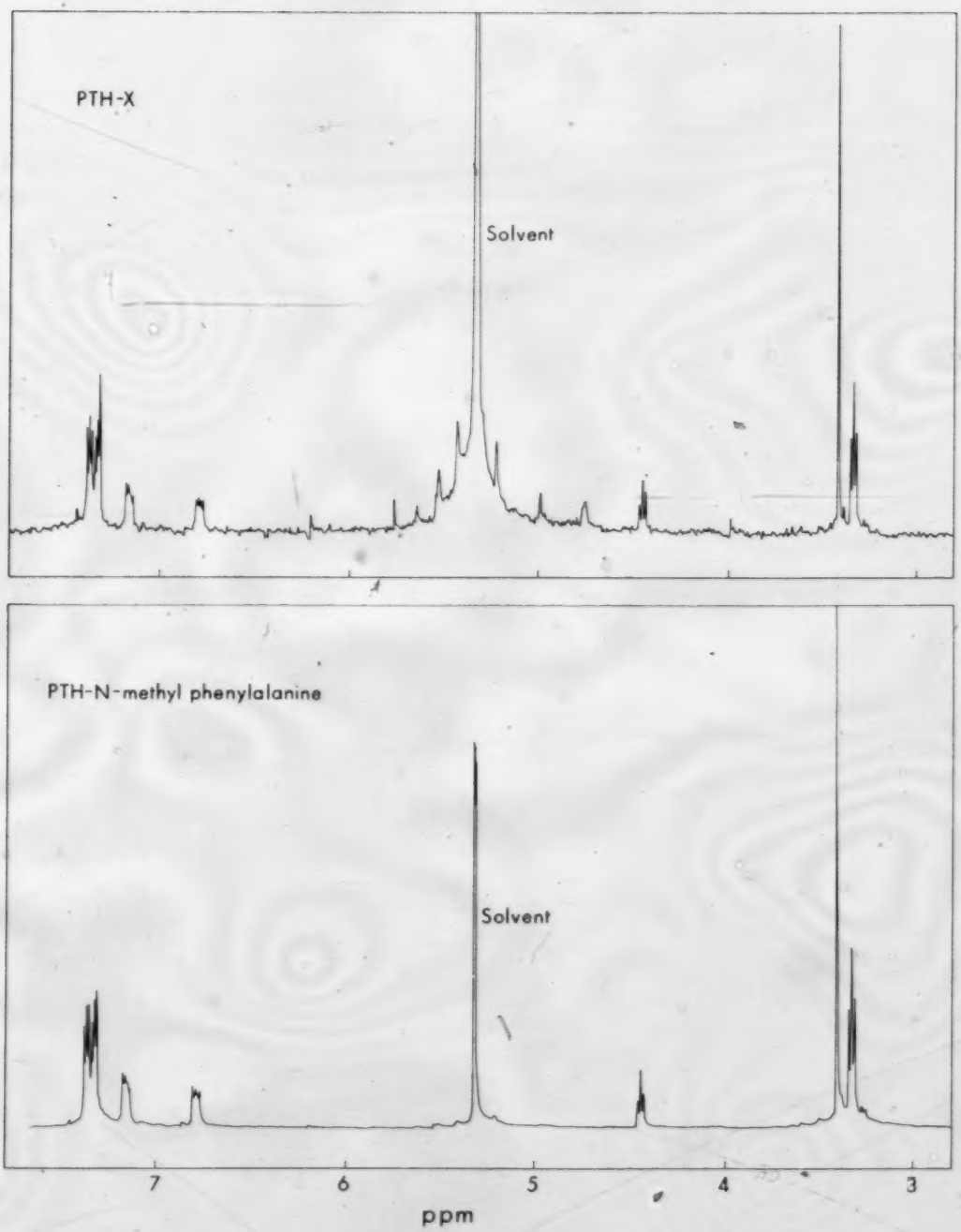
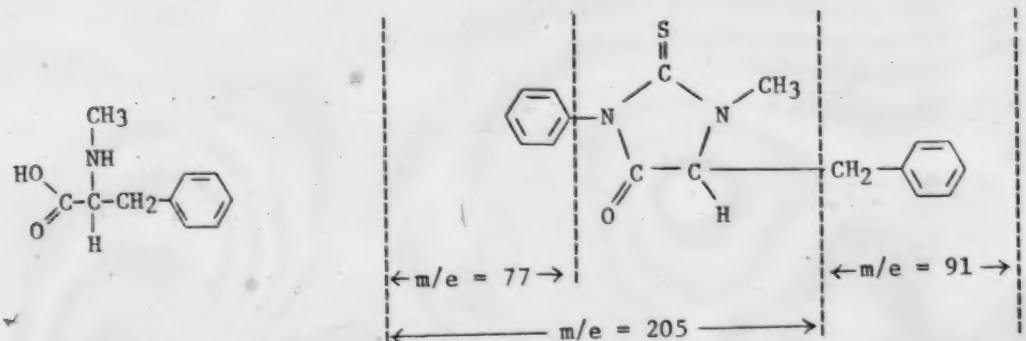

PTH-N-methyl phenylalanine
PTH-X
Relative Intensity
100
80
60
40
20
0
50 60 80 100 120 140 160 180 200 220 240 260 280 300
m/e

FIGURE 26. PMR spectra of PTH-X and PTH-N-methylphenylalanine.

Both samples, 0.2 and 1.0 mg respectively, were washed with 99.8% D_2O and dried in vacuo (2X) before being dissolved in 0.4 ml CD_2Cl_2 - d_2. The spectra were recorded with a Bruker HX270 super-conducting NMR spectrometer at 270 MHz and ambient temperature. Comparative peaks were adjusted to similar height. Chemical shifts are reported in ppm downfield from tetramethylsilane.


PTH-X
Solvent
PTH-N-methyl phenylalanine
Solvent
7
6
5
4
3
ppm

and PTH-X is shown in Figure 25. A molecular ion of m/e = 296 and major fragments of m/e = 205, 91 and 77 are evident in both spectra. The fragmentation pattern can be correlated to the structure of N-methyl-phenylalanine as shown in the following diagram:



N-methyl-phenylalanine

PTH-N-methylphenylalanine

Similarly, Figure 26 provides a comparison of the compounds' PMR spectra. The peaks downfield from 6.7 to 7.4 ppm represent the two phenyl rings in the PTH-derivatives and are typical of all the PTH-amino acids used in this study. The peak at 4.45 ppm is due to the α-carbon proton while the peak at 3.33 ppm is due to the β-carbon protons. The singlet at 3.42 ppm is indicative of the N-methyl protons since C-methyl protons usually peak upfield from this value. The ratio of protons in the peaks reading from downfield to upfield, as determined by planimetry of the 10X expanded peaks, was 5:3:2:1:3:2.

Ultraviolet absorption spectra of the compounds showed that both have a high $\varepsilon_{245}/\varepsilon_{269}$ ratio of 0.64 compared with values around 0.4 for PTH derivatives of other amino acids (Figure 27), with the exceptions of 0.72 for PTH-N^{ε}-phenylthiocarbamyl-lysine and 0.67 for PTH-proline (Edman, 1970). In addition the ε_{269} molar absorption coefficient

for the synthetic compound is lower at 13,500 than the usual value of around 16,000 for these amino acid derivatives (Edman, 1970).

N-methylphenylalanine is difficult to detect under the usual conditions employed in amino acid analysis. However using the method of Coggins and Benoiton (1970) in which a halved buffer and ninhydrin flow through the reaction coil, resulting in a doubled reaction time with ninhydrin, 1 molecule of N-methylphenylalanine was detected for every 12.6 molecules of isoleucine which is close to the predicted value of 1/14 (see Chapter V).

Prior to sequencing the first twenty-two amino acids at the N-terminus using the automated sequenator, attempts to identify the N-terminal amino acid using the dansyl chloride procedure of Gray (1972) failed. While O-DNS-tyrosine and ε-DNS-lysine could be visualized after thin layer chromatography of the hydrolyzed, dansylated protein, indicating the reaction had occurred, no spot corresponding to a possible dansylated N-terminus residue could be found. On hindsight, this result is not surprising since DNS-N-methylphenylalanine is probably destroyed during acid hydrolysis in much the same way that N-methylphenylalanine cannot be regenerated from the PTH-derivative under acidic or basic conditions.

3. C-terminal analysis

Carboxypeptidase A treated with DFP (diisopropylfluorophosphate) (CPA) was obtained from Worthington Biochemicals and prepared as described by Ambler (1967). A 25 µl suspension (1.25 mg protein) was diluted with 1.0 ml water in a 3-ml conical centrifuge tube and centrifuged for 5 min at 2000 x g. The supernatant was discarded and the pellet was suspended in 0.1 ml 1% (w/v) sodium bicarbonate and chilled in

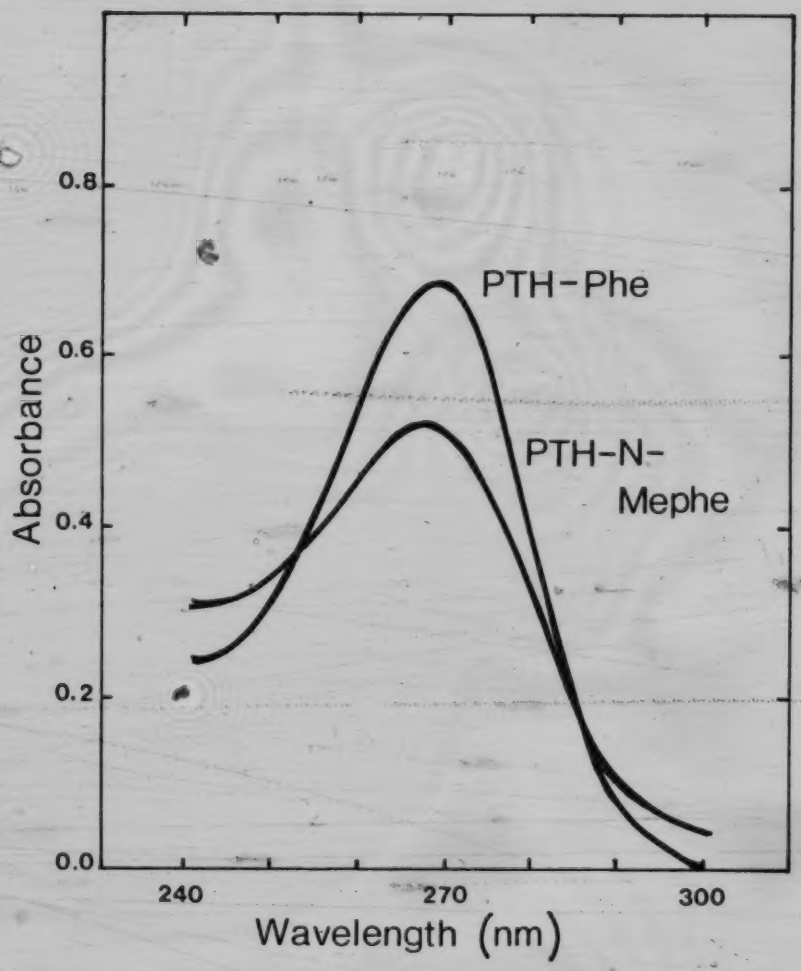


FIGURE 27. UV absorption spectrum of PTH-phenylalanine and PTH-N-methylphenylalanine.

The spectrum of PTH-phenylalanine and PTH-N-methylphenylalanine dissolved in absolute ethanol was recorded between wavelengths 240 - 300 nm using a Cary 15 recording spectrophotometer.

an ice bath. Sodium hydroxide (0.1 N) was added with thorough mixing to dissolve the protein. The pH was adjusted to 8.5 with 0.1 N HCl and the volume was adjusted to 1.25 ml (1 mg protein/ml) with 0.2 M N-ethylmorpholine acetate pH 8.5. The enzyme preparation was used immediately. Salt-free PAK/2PfS pili was dissolved in 1.0 ml 0.2 M N-ethylmorpholine, pH 8.5 and 0.1% SDS. The solution was heated to 100° for 5 min and was cooled to room temperature. CPA (2.5 nmol or 80 µl of prepared enzyme solution) was added to give a substrate to enzyme ratio of 40, and the mixture was incubated at room temperature. Appropriate enzyme controls were done simultaneously. At various time intervals (0.25, 0.5, 1.0, 2.0 and 4.0 h) 0.2 ml of sample were acidified to pH 2.0 with acetic acid and clarified by low speed centrifugation. The supernatant was lyophilized and analyzed for amino acid content on a Durrum D500 acid analyzer. The results are shown in Figure 28.

From the relative rates of amino acids released it can be seen that serine is probably the C-terminus and lysine is the penultimate amino acid. Since the initial rates of release for both amino acid are nearly the same, the order of these amino acids could be reversed or there may be two populations of pilin molecule with either serine or lysine at the C-terminal. Adjusting the substrate to enzyme ratio to 10 or 200 or carrying out the reaction at 37° gave identical results. Prolonged exposure of pilin to CPA (16 h) failed to yield an antipenultimate amino acid indicating the presence of a non-reactive amino acid.

4. Cleavage with cyanogen bromide

Purified PAK/2PfS pilin was cleaved into as many as 4 peptides by cyanogen bromide according to the method of Steers *et al.* (1965). 50 mg of dry PAK/2PfS pili (64 A_{280} units) were dissolved in 14 ml of 100% formic acid which was diluted to 70% by the addition of 6 ml of water.

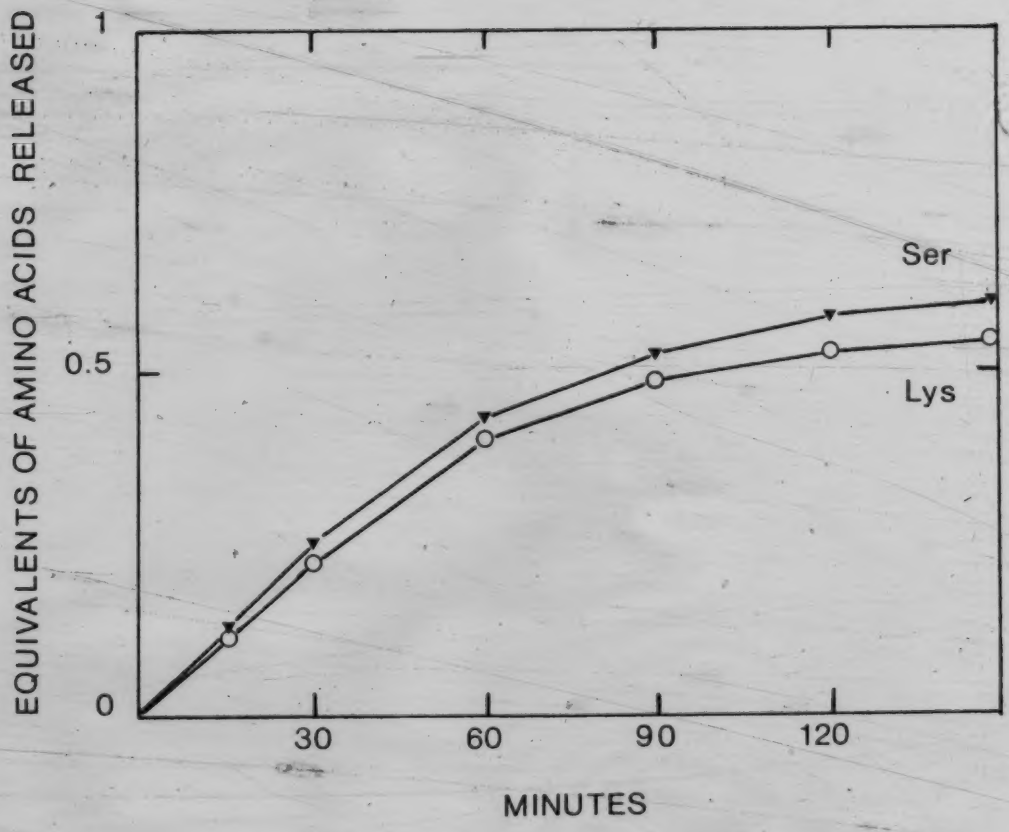


FIGURE 28. Treatment of PAK pilin with carboxypeptidase A.

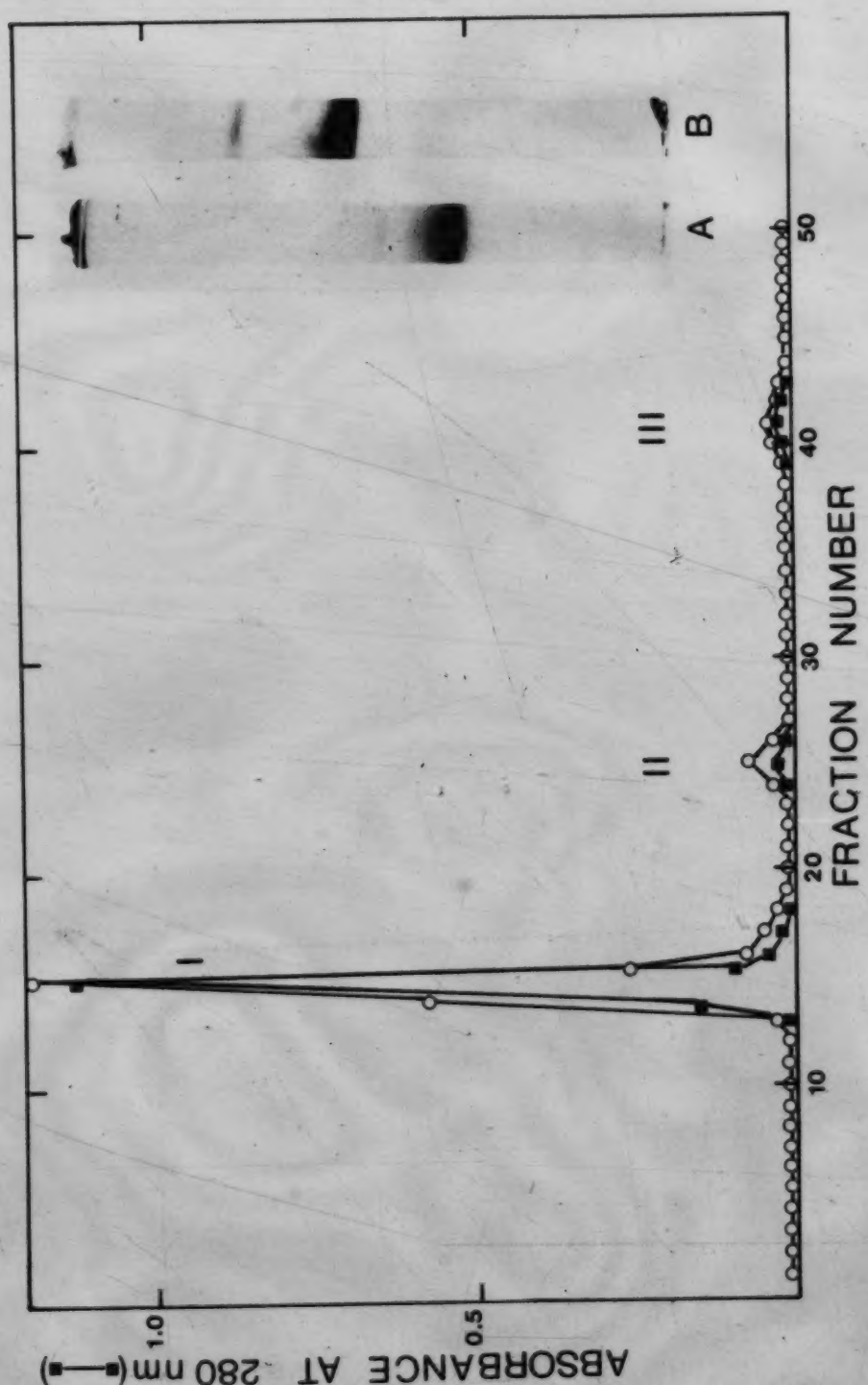
The substrate/enzyme ratio was 40. The reaction took place at room temperature in the presence of 0.1% SDS at pH 8.5.

Crystalline cyanogen bromide (Eastman Organic Chemicals) was added to give methionine:CNBr ratio of 1:300 based on three methionine residues/ pilin molecule. The flask was tightly stoppered and the reaction was allowed to proceed at room temperature for 24 hours. The reaction was stopped by diluting the reaction mixture to 10% formic acid with water and the solution was shell frozen and lyophilized to complete dryness. Triplicate samples containing 0.5 mg of cleaved protein each were hydrolyzed for amino acid analysis. Prior to analysis, the homoserine lactone residues were converted to homoserine by incubating the previously hydrolyzed samples in sealed tubes in 0.5 ml of pyridine acetate buffer, pH 6.5, at 105° for one hour. The samples were dried by lyophilization and analyzed for amino acids using a Durrum D500 amino acid analyzer programmed to detect homoserine. While no methionine could be detected, 2.9 homoserine residues were present per pilin molecule, indicating the reaction was close to completion. A 25 µg sample of the reaction products was run on a 12.5% polyacrylamide gel (0.1% SDS). A second gel contained 25 µg of unreacted PAK pilin. The gels are shown in Fig. 29, where it may be seen that the CNBr reaction produced one principal fragment of molecular weight 12,000 daltons, and smaller amounts of material at 13,000, 14,000 and 16,000 daltons. These smaller bands represent incompletely cleaved intermediates in the CNBr reaction. The smaller peptides produced by this reaction were not detectable on the gel.

The CNBr fragments were separated by gel filtration using a Sephadex G-50 superfine (Pharmacia, Sweden) column. A portion of reacted material (30 mg) was dissolved in a minimum volume of 50% formic acid and was diluted to 5% formic acid to give a total volume of 20 ml. The

FIGURE 29. Separation of CNBr fragments of PAK pilin by gel filtration.

35 mg of PAK pilin treated with cyanogen bromide at a ratio of 300:1 was dissolved in 20 ml 5% formic acid and run on a Sephadex G-50 superfine column (2.5 cm x 100 cm) at 20°. The eluant was 5% formic acid, the flow rate 25 ml/h and the void volume was 60 ml. Each of the resultant 5 ml fractions was assayed for absorbance at 280 nm (■-■) and ninhydrin positive material (○-○). A gel of 25 µg of material before (B) and after (A) treatment with cyanogen bromide is shown in the inset.


ABSORBANCE AT 280 nm (■—■)
ABSORBANCE AT 570 nm (○—○)
1.0
0.5
10
20
30
40
50
FRACTION NUMBER
I
II
III
A
B

sample was applied directly to a 2.5 x 100 cm column of Sephadex G-50 superfine equilibrated with 5% formic acid which was also used to elute the CNBr fragments from the column. The flow rate was 25 ml/h. The absorbance at 280 nm was measured using a Beckman DBG spectrophotometer. The small peptides were barely identifiable by absorbance at 280 nm because of a probable lack of aromatic amino acids in them and the samples were assayed for protein using the ninhydrin colorimetric assay (Hirs, 1967c). The results are shown in Figure 29, where it may be seen that the CNBr reaction products separated into three peaks. The fractions in each of the three peaks were pooled and lyophilized to dryness: 0.5 mg of peaks I and II and 0.2 mg of peak III were hydrolyzed for analysis using the Durrum D500 amino acid analyzer. The results are shown in Table 7 and are calculated on the basis of a common denominator for each of the values for the amino acids found in each sample. No determination for tryptophan or cysteine was done at this time. The total for any amino acid from peaks I, II and III approximates very closely the previously determined number/pilin molecule, indicating that cyanogen bromide cleaves the pilin molecule into at least three fragments. Because the sample size was very small due to the insolubitiy of freeze-dried fractions of peak II and III, these results are to be considered as very preliminary.

Inspection of the sequence at the N-terminus indicates that a peptide containing 7 amino acids should result from cyanogen bromide cleavage, namely N-methyl-Phe-Thr-Leu-Ile-Glu-Leu-Met (as homoserine). N-methylphenylalanine, when special conditions for its detection were employed (Coggins and Benoiton, 1970), was barely detectable in peak III indicating that a mixture of two small peptides probably existed,

TABLE 7

Amino Acid Analysis of CNBr Fragments of PAK/2PfS Pilin

Amino Acid	Pilin (18,620)	Peak I (11,900)	Peak II (4000)	Peak III (1900)	N-terminus[1]	C-terminus[2]
Asp	15	9-10	4	2	0	2
Thr	15	9	4	1	1	0
Ser	10	8	2	1	0	1
Glx	15	11	3-4	2-3	1	1-2
Pro	10	6-7	n.d.	n.d.	0	?
Gly	18	10-12	6	3	0	3
Ala	24	15	4-5	2	0	2
Val	9	9	0	0	0	0
Ile	12	7-8	2-3	1	1	0
Leu	14	9-10	2-3	1-1.5	2	0
Tyr	3	3	0	0	0	0
Phe	3	1	1	0	0	0
Lys	15	8	6-7	1	0	1
Arg	4	3	1	0	0	0
N-MePhe	1	0	n.d.	+[4]	1	0
HS(Met)	3	1	1.0	?	1	0
Trp	2	n.d.[3]	n.d.	n.d.	0	0
Cys	4	n.d.	n.d.	n.d.	0	0

[1]The N-terminus amino acid composition is deduced from the sequence of the first seven amino acid residues (H_2N-N-MePhe-Thr-Leu-Ile-Glu-Leu-Met-).

[2]The C-terminus amino acid composition is tentatively determined by subtracting the amino acid residues for the N-terminus from the amino acid residues found in peak III.

[3]Not determined (n.d.).

[4]+, identified but not quantitated.

	1				5				10	
M. nonliquefaciens*	(N-MePhe)	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Ile	Ala
N. gonorrhoeae*	N-MePhe	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Ile	Ala
P. aeruginosa K	N-MePhe	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Val	Ala
E. coli type 1*	Ala	Ala	Thr	Thr	Val	Asn	Gly	Gly	Thr	Val	His

	12			15					20		
M. nonliquefaciens	Ile	Ile	Gly	Ile	Leu	Ala	Ala	Ile	Ala	Leu	Pro
N. gonorrhoeae	Ile	Val	Gly	Ile	Leu	Ala	Ala	Val	Ala	Leu	Pro
P. aeruginosa K	Ile	Ile	Gly	Ile	Leu	Ala	Ala	Ile	Ala	Ile	Pro
E. coli type 1	Phe	Lys	Gly	Glu	Val	Val	Asn	Ala	Ala	X	Ala

* Hermodson, Chen & Buchanan (1978)

FIGURE 30. The amino acid sequence at the amino terminus of several pilin molecules.

one corresponding to the N-terminal peptide above and one containing 2 Asp, 1 Ser, 1 Glu, 3 Gly, 2 Ala and 1 Lys. The absence of methionine (homoserine) and the presence of lysine and serine, the amino acids detected at the C-terminus, indicates that this could be the C-terminal peptide. The amounts of homoserine detected in peaks II and III were difficult to determine because of the small sample size.

C. CONCLUSION

Preliminary chemcial analyses on PAK pilin have established the sequence of the first twenty-two amino acids at the amino terminus as well as the identity of the N-terminal amino acid, N-methylphenylalanine. This sequence was very similar to the sequence at the N-terminus of pilin derived from Moraxella nonliquefaciens (Froholm and Sletten, 1977) and Neiserria gonorrhoeae (Hermodson et al., 1978). As shown in Figure 30, all three amino terminal sequences are highly homologous. The only differences are Val-Ile exchanges at positions 10, 13 and 19, and an Ile-Leu exchange in position 21. While Frøholm and Sletten (1977) did not identify the N-terminal residue, their evidence suggests that it is N-methylphenylalanine. In the case of M. nonliquefaciens and P. aeruginosa K pilin, a single N-terminal residue was found while 50% of N. gonorrhoeae pilin subunits lacked the N-methylphenylalanine amino terminal residue.

N-methylphenylalanine has not yet been detected in non-pilus proteins although it has been shown to be a constituent of the peptide antibiotic Staphlomycin S (Vanderhaeghe and Parmentier, 1960). Pettigrew and Smith (1977) have reported finding dimethylproline at the amino terminus of Crithidia oncopelti cytochrome C557, while Chen et al.

(1977) have shown that certain ribosomal proteins of E. coli contain N-monomethylalanine or N-monomethylmethionine as amino terminal residues. The biological significance of α-methylated N-terminal amino acid residues is not clear at this time. The N-methylphenylalanine in PAK pilin does not act as a blocking group to sequence analysis since the Edman degradation in subsequent cycles proceeded normally with no obvious drop in efficiency between the first and second cycles. The other common secondary amino in sequence work, proline, does not interfere with the process.

The occurrence of a highly hydrophobic, common sequence at the amino terminus of pilin provided by three relatively unrelated microorganisms suggests this common sequence may be important to pilus function. These hydrophobic amino terminal sequences are reminiscent of the hydrophobic extensions found on precursors of secretory proteins in mammalian cells (Devillers-Thiery et al., 1975). According to the signal hypothesis of Blobel and Dobberstein (1975) this hydrophobic extension facilitated transfer of the protein through the membrane in the process of which, it was cleaved off the protein. Thus the hydrophobic amino terminal portion of the pilin molecule may provide a pilot function which facilitates the transport of pilin subunits from the cell interior to the outer membrane. Once the pilin subunits are assembled into pili, the highly hydrophobic amino terminal sequence would presumably be buried within the subunit or involved in subunit-subunit interactions.

Evidence already exists that pilin pools are located in the outer membrane of bacteria. Beard and Connally (1974) have detected F-type R pilin molecules in the outer membrane of E. coli and suggested that

this is the site for pilus assembly. Recently Olsen *et al.* (1977) have speculated on the presence of pilin molecules in the outer membrane of *P. aeruginosa* which act as receptor sites for PRD-1 phage particles.

Although it is tempting to suggest that a hydrophobic amino terminus is a universal feature of pilus systems, the amino terminal sequence of type 1 pilin of *E. coli* (Hermodson *et al.*, 1978) bears no homology to the three sequences previously discussed nor does it contain N-methylphenylalanine.

Thus the structure-function relationships of pili are probably far more complicated than a system such as flagella whose structure and chemistry is fairly constant throughout the microbial world (Iino, 1968). The study of the chemistry of a number of different types of pili may help to elucidate the mechanism of various pilus functions.

This investigation has shown that the PAK pilus is an interesting system in its own right and ought not to take a second place to a better defined system such as the F pilus of *E. coli*. The finding that PAK pilin is similar in amino acid composition and N-terminal sequence to a pilin isolated from a pathological strain (*N. gonorrhoeae*) is very interesting. The gonococcal pilus contributes to the pathogenicity of this strain by being involved in the adhesion of the bacterial cell to mammalian cells (Punsalung and Sawyer, 1973). While the sequencing of PAK pilin is in progress at this time, information is needed concerning the genetic origin of PAK pili, its involvement, if any, in adhesion to mammalian cells or in bacterial conjugation, and the mechanism of pilus assembly both *in vitro* and *in vivo*. Since PAK pili are both abundant and easily purified and techniques for handling *Pseudomonas aeruginosa* are almost as advanced as those for *E. coli*, progress in this area should be forthcoming.

BIBLIOGRAPHY

Abodeely, R.A., E. Palmer, L.A. Lawson and C.C. Randall (1971) Virology 44:146.

Achtman, M. (1973) Genet. Res. Camb. 21:67.

Achtman, M. and R. Helmuth (1975) In Microbiology - 1974. Edited by D. Schlessinger. p. 95. American Society for Microbiology, Washington, D.C.

Achtman, M., N. Kennedy and R. Skurray (1977) Proc. Natl. Acad. Sci. U.S.A. 74:5104.

Achtman, M., N.S. Willetts and A.J. Clark (1971) J. Bacteriol. 106: 529.

Achtman, M., N.S. Willetts and A.J. Clark (1972) J. Bacteriol. 110: 831.

Albertsson, P.-A. (1960) Partition of Cell Particles and Macromolecules . John Wiley and Sons, New York.

Alfaro, G. and N.S. Willetts (1972) Genet. Res. Camb. 20:279.

Ambler, R.P. (1967) Methods in Enzymol. 11:155.

Argetsinger, J.E.A. and G.N. Gussin (1966) J. Mol. Biol. 21:421.

Anderson, T.F. (1958) Cold Spring Harbor Symp. Quant. Biol. 23:47.

Ashwell, G. (1957) Methods in Enzymol. 3:73.

Beard, J.P. (1973) Abstracts, 68th Ordinary Meeting of the Society of General Microbiology, Canterbury, England. p. 11.

Beard, J.P. and J.C. Connally (1975) J. Bacteriol. 122:59.

Beard, J.P., T.G.B. Howe and M.H. Richmond (1972a) J. Mol. Biol. 66: 311.

Beard, J.P., T.G.B. Howe and M.H. Richmond (1972b) J. Bacteriol. 111: 814.

Blobel, G. and B. Dobberstein (1975) J. Cell. Biol. 67:835.

Bradley, D.E. (1965) J. Roy. Microsc. Soc. 84:257.

Bradley, D.E. (1966) J. Gen. Microbiol. 45:83.

Bradley, D.E. (1972a) Genet. Res. 19:39.

Bradley, D.E. (1972b) Biochem. Biophys. Res. Commun. 47:142.

Bradley, D.E. (1972c) Biochem. Biophys. Res. Commun. 47:1080.

Bradley, D.E. (1972d) J. Gen. Microbiol. 72:303.

Bradley, D.E. (1973a) Can. J. Microbiol. 19:623.

Bradley, D.E. (1973b) J. Virol. 12:1139.

Bradley, D.E. (1974a) Biochem. Biophys. Res. Commun. 57:893.

Bradley, D.E. (1974b) Virology 58:149.

Bradley, D.E. (1975a) Biochem. Biophys. Res. Commun. 64:918.

Bradley, D.E. (1975b) Proceedings of the Second International Pili Conference (in press).

Bradley, D.E. (1977a) In Microbiology - 1977. Edited by D. Schlessinger. p. 127. American Society for Microbiology, Washington, D.C.

Bradley, D.E. (1977b) J. Gen. Microbiol. 102:349.

Bradley, D.E. and D.R. Cohen (1976) J. Gen. Microbiol. 97:91.

Bradley, D.E. and T.L. Pitt (1974) J. Gen. Virol. 24:1.

Bradley, D.E. and T.L. Pitt (1975) J. Hyg. 74:419.

Bradley, D.E. and E.L. Rutherford (1975) Can. J. Microbiol. 21:152.

Brauer, A.W., M.N. Margulies and E. Haber (1975) Biochemistry (U.S.) 14:3029.

Bridgen, J., A.P. Graffeo, B.L. Karger and M.D. Waterfield (1975) In Instrumentation in Amino Acid Sequence Analysis. Edited by R.N. Perham. p. 111. Academic Press, London.

Brinton, C.C. (1965) Trans. N.Y. Acad. Sci. 27:1003.

Brinton, C.C. (1971) Crit. Rev. Microbiol. 1:105.

Brinton, C.C. and H. Beer (1967) In Molecular Biology of Viruses. Edited by J.S. Colter and W. Paranchych. p. 251. Academic Press, New York.

Brinton, C.C., P. Gemski and J. Carnahan (1964) Proc. Natl. Acad. Sci. U.S.A. 52:776.

Caro, L.G. and M. Schnös (1966) Proc. Natl. Acad. Sci. U.S.A. 56:126.

Cavalli-Sforza, L.L. and E.M. Lederberg (1953) J. Gen. Microbiol. 8:89.

Chandler, P.M. and V. Krishnapillai (1974) Genet. Res. 23:239.

Chen, P.S., T.Y. Toribara and H. Warner (1956) Anal. Chem. 28:1756.

Chen, R., J. Brosius and B. Wittman-Liebold (1977) J. Mol. Biol. 111: 173.

Clark, J.T. (1964) Ann. N.Y. Acad. Sci. 121:428.

Coggins, J.R. and N.L. Benoiton (1970) J. Chromatog. 52:251.

Crawford, E.M. and R.F. Gesteland (1964) Virology 22:165.

Curtiss, R., L.G. Caro, D.P. Allison and D.R. Stallions (1969) J. Bacteriol. 100:1091.

Curtiss, L.K. and R.G. Krueger (1974) J. Virol. 14:503.

Cutting, J.R. and T.F. Roth (1973) Anal. Biochem. 54:386.

Danziger, R.E. and W. Paranchych (1970a) Virology 40:547.

Danziger, R.E. and W. Paranchych (1970b) Virology 40:554.

Date, T., M. Inuzuka and M. Tomoeda (1978) Biochemistry (U.S.) 16:5579.

Datta, N., R.W. Hedges, E.J. Shaw, R.B. Sykes and M.H. Richmond (1971) J. Bacteriol. 108:1244.

Datta, N., A.M. Lawn and E. Meynell (1966) J. Gen. Microbiol. 45:365.

Davis, B.D. (1950) J. Bacteriol. 60:507.

Dennison, S. (1972) J. Bacteriol. 109:416.

Dennison, S. and R.W. Hedges (1972) J. Hygiene 70:55.

De Pamphilis, M.L. and J. Adler (1971) J. Bacteriol. 105:376.

Devillers-Thiery, A., T. Kindt, G. Scheele and G. Blobel (1975) Proc. Natl. Acad. Sci. U.S.A. 72:5016.

Donelian, J. (1972) M.Sc. Thesis, University of Alberta, Edmonton.

Dowman, J.E. and G.G. Meynell (1970) Molec. Gen. Genet. 109:57.

Duguid, J.P., E.S. Anderson and I. Campbell (1966) J. Pathol. Bacteriol. 92:107.

Edman, P. (1970) In Protein Sequence Determination. Edited by S.B. Needleman. p. 211. Springer-Verlag, New York.

Edman, P. and G. Begg (1967) Eur. J. Biochem. 1:80.

Egawa, R. and Y. Hirota (1962) Jap. J. Genet. 37:66.

Fairbanks, G., T.L. Steck and D.F.J. Wallach (1971) Biochemistry 10: 2606.

Falkow, S. and L.S. Baron (1962) J. Bacteriol. 98:1598.

Feary, T.W., E. Fisher and T.N. Fisher (1964) J. Bacteriol. 87:196.

Fenwick, R.G. and R. Curtiss (1973a) J. Bacteriol. 116:1213.

Fenwick, R.G. and R. Curtiss (1973b) J. Bacteriol. 116:1224.

Finnegan, D. and N.S. Willetts (1973) Mol. Gen. Genet. 127:307.

Fiske, C.H. and Y. SubbaRow (1925) J. Biol. Chem. 66:375.

Frøholm, L.O. and K. Sletten (1977) FEBS Letters 73:29.

Frost, L.S., M. Carpenter and W. Paranchych (1978) Nature (London) 271: 87.

Frost, L. and W. Paranchych (1977) J. Bacteriol. 131:259.

Gasson, M.J. and N.S. Willets (1975) J. Bacteriol. 122:518.

Gasson, M.J. and N.S. Willets (1977) J. Bacteriol. 131:413.

Gesteland, R.F. and H. Boedtker (1964) J. Mol. Biol. 8:496.

Goldschmidt, M.C. and O. Wyss (1967) J. Gen. Microbiol. 47:421.

Goodwin, T.W. and R.A. Morton (1946) Biochem. J. 40:628.

Griffith, I.P. (1972) Anal. Biochem. 46:402.

Grindley, J.N. and E.S. Anderson (1971) Genet. Res. 17:267.

Grindley, N.D.F., J.N. Grindley, H.R. Smith and E.S. Anderson (1973) Molec. Gen. Genet. 120:27.

Hayes, W. (1952a) Nature 169:118.

Hayes, W. (1952b) Nature 169:1017.

Hayes, W. (1953) J. Gen. Microbiol. 8:72.

Hayes, W. (1964) The Genetics of Bacteria and their Viruses. Blackwell Sci. Publications, Oxford, England.

Hedges, R.W. (1972) J. Gen. Microbiol. 72:407.

Hedges, R.W. (1974) J. Gen. Microbiol. 81:171.

Hedges, R.W. and N. Datta (1971) Nature (London) 224:220.

Hedges, R.W. and N. Datta (1972) J. Gen. Microbiol. 71:403.

Hedges, R.W., N. Datta, T.N. Coetzee and S. Dennison (1973) J. Gen. Microbiol. 77:249.

Hedges, R.W and A.E. Jacob (1974) Molec. Gen. Genet. 132:31.

Helmuth, R. and M. Achtman (1975) Nature (London) 257:652.

Hermodson, M.A., K.C.S. Chen and T.M. Buchanan (1978) Biochemistry (U.S.) 17:442.

Hirs, C.H.W. (1967a) Methods in Enzymol. 11:59.

Hirs, C.H.W. (1967b) Methods in Enzymol. 11:197.

Hirs, C.H.W. (1967c) Methods in Enzymol. 11:325.

Hirs, C.H.W. (1967d) Methods in Enzymol. 11:411.

Holloway, B.W. (1969) Bacteriol. Rev. 33:419.

Iino, T. (1969) Bacteriol. Rev. 33:454.

Ippen-Ihler, K., M. Achtman and N.S. Willetts (1972) J. Bacteriol. 110: 857.

Ippen, K.A. and R.C. Valentine (1965) Biochem. Biophys. Res. Commun. 21:21.

Ippen, K.A. and R.C. Valentine (1967) Biochem. Biophys. Res. Commun. 27:674.

Ishibashi, M. (1967) J. Bacteriol. 93:379.

Iyobe, S., S. Mitsuhashi and H. Umezawa (1971) J. Bacteriol. 108:946.

Jacob, F. and E.L. Wollman (1961) Sexuality and the Genetics of Bacteria . Academic Press, New York.

Jacobson, A. (1972) J. Virol. 10:835.

Jacoby, G.A. (1977) In Microbiology - 1977, Edited by Schlessinger, p. 119. American Society for Microbiology, Washington, D.C.

Khatoon, H., R.V. Iyer and V.N. Iyer (1972) Virology 48:145.

Knolle, P. (1967a) Biochem. Biophys. Res. Commun. 27:81.

Knolle, P. (1967b) Zentralb. Bakteriol. I. Abtlg. Orig. 202:417.

Krahn, P.M., R. O'Callaghan and W. Paranchych (1972) Virology 47:628.

Krahn, P.M. and W. Paranchych (1971) Virology 43:533.

Lawn, A.M. (1966) J. Gen. Microbiol. 45:377.

Lawn, A.M. (1967) Nature (London) 214:1151.

Lawn, A.M. and E. Meynell (1970) J. Hyg. Camb. 68:683.

Lawn, A.M. and E. Meynell (1972) Nature (London) 235:441.

Lawn, A.M., G.G. Meynell, E. Meynell and N. Datta (1967) Nature (London) 216:343.

Lederberg, J. (1955) J. Cellular Comp. Physiol. 45:75 (suppl. 2).

Lederberg, J. (1956) J. Bacteriol. 71:497.

Lederberg, J. (1957) Proc. Natl. Acad. Sci. 43:1060.

Lederberg, J., L.L. Cavalli and E.M. Lederberg (1952) Genetics 37:720.

Lederberg, J. and E.L. Tatum (1946) Cold Spring Harbor Symp. Quant. Biol. 11:413.

Liu, T.-Y. and Y.H. Chang (1971) J. Biol. Chem. 246:2842.

Loeb, T. and N.D. Zinder (1961) Proc. Natl. Acad. Sci. U.S.A. 47:282

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) J. Biol. Chem. 193:265.

Marvin, D.A. (1966) J. Mol. Biol. 15:8.

Marvin, D.A. and B. Hohn (1969) Bacteriol. Rev. 33:172.

McDermott, J.R. and N.L. Benoiton (1973) Can. J. Chem. 51:1915.

Meynell, E. (1973) J. Bacteriol. 113:502.

Meynell, E. and N. Datta (1965) Nature (London) 207:884.

Meynell, E., G.G. Meynell and N. Datta (1968) Bacteriol. Rev. 32:55.

Meynell, G.G. and A.M. Lawn (1967a) Genet. Res. 9:359.

Meynell, G.G. and A.M. Lawn (1967b) Genet. Res. 10:323.

Meynell, G.G.and A.M. Lawn (1968) Nature (London) 217:1184.

Miki, T., Y. Sakane and T. Horiuchi (1969) Jap. J. Genet. 44:398.

Miki, T., Y. Sakane and T. Horiuchi (1970) Jap. J. Genet. 45:482.

Miller, J.H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, New York.

Minkley, E.G. and K. Ippen-Ihler (1977) J. Bacteriol. 129:1613.

Minkley, E.G., S. Polen, C.C. Brinton and K. Ippen-Ihler (1976) J. Mol. Biol. 108:111.

Mitsuhashi, S., K. Harada and H. Hashimoto (1960) Jap. J. Exp. Med. 30: 179.

Moore, S. (1963) J. Biol. Chem. 238:235.

Nishimura, Y., M. Ishibashi, E. Meynell and Y. Hirota (1967) J. Gen. Microbiol. 49:89.

Novick, R.P., R.C. Clowes, S.N. Cohen, R. Curtiss, N. Datta and S. Falkow (1976) Bacteriological Rev. 40:168.

Novotny, C.P., J. Carnahan and C.C. Brinton (1969a) J. Bacteriol. 98: 1294.

Novotny, C.P. and P. Fives-Taylor (1974) J. Bacteriol. 117:1306.

Novotny, C.P., P. Fives-Taylor and K. Lavin (1972) J. Bacteriol. 112: 1083.

Novotny, C.P. and K. Lavin (1971) J. Bacteriol. 107:671.

Novotny, C.P., W.S. Knight and C.C. Brinton (1968) J. Bacteriol. 95: 314.

Novotny, C., E. Raizon, W.S. Knight and C.C. Brinton (1969b) J. Bacteriol. 98:1307.

O'Callaghan, R., R. Bradley and W. Paranchych (1973a) Virology 54:476.

O'Callaghan, R.J., L. Bundy, R. Bradley and W. Paranchych (1973b) J. Bacteriol. 115:76.

Ohki, M. and H. Ozeki (1968) Molec. Gen. Genet. 103:37.

Ohki, M. and J. Tomizawa (1968) Cold Spring Harbor Quant. Symp. Biol. 33:651

Ohtsubo, E., Y. Nishimura and Y. Hirota (1970) Genetics 64:173.

Olsen, R.H. and P. Shipley (1973) J. Bacteriol. 113:772.

Olsen, R.H., J. Siak and R.H. Gray (1974) J. Virol. 14:689.

Olsen, R.H., J.-S. Siak and P.L. Shipley (1977) In Microbiology - 1977. Edited by D. Schlessinger. p. 138. American Society for Microbiology, Washington, D.C.

Olsen, R.H., and D.D. Thomas (1973) J. Virol. 12:1560.

Ou, J.T. (1973) J. Bacteriol. 114:1108.

Ou, J.T. and T.F. Anderson (1970) J. Bacteriol. 102:648.

Ou, J.T. and T.F. Anderson (1972) J. Bacteriol. 111:177.

Ozeki, H. (1965) Zbt. Bakt. I. Abt. Orig. 196:160.

Paranchych, W. (1966) Virology 28:90.

Paranchych, W. (1975) In RNA Phages. Edited by N.D. Zinder. pp. 85-111. Cold Spring Harbor Laboratory, New York.

Paranchych, W., S.K. Ainsworth, A.J. Dick and P.M. Krahn (1971) Virology 45:615.

Paranchych, W. and A.F. Graham (1962) J. Cellular Comp. Physiol. 60:199.

Paranchych, W., P.M. Krahn and R.D. Bradley (1970) Virology 41:465.

Pettigrew, G.W. and G.M. Smith (1977) Nature (London) 265:661.

Pinney, R.J. and J.T. Smith (1974) J. Gen. Microbiol. 82:415.

Pisano, J.J. and T.J. Bronzert (1969) J. Biol. Chem. 244:5597.

Punsalung, A.P. and W.D. Sawyer (1973) Infect. Immun. 8:255.

Reiner, A.M. (1974) J. Bacteriol. 119:183.

Robertson, J.N., P. Vincent and M. Ward (1977) J. Gen. Microbiol. 102: 169.

Romero, E. and E. Meynell (1969) J. Bacteriol. 97:780.

Shahrabadi, M.S., L.E. Bryan and H.M. Van Den Elzen (1975) Can. J. Microbiol. 21:592.

Silverman, P.M. and R.C. Valentine (1969) J. Gen. Virol. 4:111.

Skurray, R.A., R.E.W. Hancock and P. Reeves (1974) J. Bacteriol. 119: 726.

Smilowitz, H. (1974) J. Virol. 13:94.

Smithies, O., D. Gibson, E.M. Fanning, R.M. Goldfliesch, J.G. Gilman and D.L. Ballantyne (1971) Biochemistry (U.S.) 10:4912.

Stahl, E. and U. Kaltenbach (1961) J. Chromatogr. 5:351.

Stallions, D.R. and R. Curtiss (1972) J. Bacteriol. 111:294.

Stanisisch, V. (1974) J. Gen. Microbiol. 84:332.

Steers, E., G.R. Craven and C.B. Anfinsen (1965) J. Biol. Chem. 240: 2478.

Steitz, J.A. (1968) J. Mol. Biol. 33:937.

Swaney, L.M., Y.-P. Liu, K. Ippen-Ihler and C.C. Brinton (1977) J. Bacteriol. 130:506.

Takeya, K. and K. Amako (1966) Virology 28:163.

Tatum, E.L. and E. Lederberg (1947) J. Bacteriol. 53:673.

To, C.M., A. To and C.C. Brinton (1975) In Abstracts of the Annual Meeting of the American Society for Microbiology. P. 259. American Society for Microbiology, Washington, D.C.

Tomoeda, M., M. Inuzuka and T. Date (1975) Prog. Biophys. Mol. Biol. 30:23.

Trenkner, E., F. Bonhoeffer and A. Gierer (1967) Biochem. Biophys. Res. Commun. 28:932.

Trevelyan, W.E., D.P. Proctor and J.S. Harrison (1950) Nature (London) 166:444.

Tzagoloff, H. and D. Pratt (1964) Virology 24:372.

Valentine, R.C., P.M. Silverman, K.A. Ippen and H. Mobach (1969) In Advances in Microbial Physiology. Edited by A. Rose and J. Wilkinson. 3:1.

Valentine, R.C. and M. Strand (1965) Science 148:511.

Valentine, R.C. and H. Wedel (1965) Biochem. Biophys. Res. Commun. 21: 106.

Valentine, R.C., H. Wedel and K.A. Ippen (1965) Biochem. Biophys. Res. Commun. 21:277.

Vanderhaeghe, J. and G. Parmentier (1960) J. Am. Chem. Soc. 82:4414.

Vapnek, D., M.B. Lipman and W.D. Rupp (1971) J. Bacteriol. 108:508.

Vapnek, D. and W.D. Rupp (1970) J. Mol. Biol. 53:287.

Vasquez, C., N. Granboulan and R.M. Franklin (1966) J. Bacteriol. 92: 1779.

Vesterberg, O. and H. Svensson (1966) Acta Chem. Scand. 20:820.

Watanabe, T. (1963) Bacteriol. Rev. 27:87.

Watanabe, T. and T. Rukasawa (1962) J. Bacteriol. 83:727.

Watanabe, T., H. Nishida, C. Ogata, T. Arai and S. Sato (1964) J. Bacteriol. 88:716.

Weber, K. and M. Osborn (1969) J. Biol. Chem. 244:4406.

Weiss, R.L. (1971) J. Gen. Microbiol. 67:135.

Wendt, L.W., K.A. Ippen and R.C. Valentine (1966) Biochem. Biophys. Res. Commun. 23:375.

Weppelman, R.M. and C.C. Brinton (1971) Virology 44:1.

Willetts, N.S. (1970) Molec. Gen. Genet. 108:365.

Willetts, N.S. (1971) Nature New Biol. 230:183.

Willetts, N.S. (1972a) J. Bacteriol. 112:773.

Willetts, N.S. (1972b) Ann. Rev. Genetics 6:257.

Willetts, N.S. (1973) Genet. Res. Camb. 21:205.

Willetts, N.S. (1974a) J. Bacteriol. 118:778.

Willetts, N.S. (1974b) Molec. Gen. Genet. 129:123.

Willetts, N.S. (1975) In Microbial Drug Resistance. Edited by S. Mitsuhashi and H. Hashimoto. University Park Press, Baltimore, Md.

Willetts, N.S. and M. Achtman (1972) J. Bacteriol. 110:843.

Willetts, N.S. and W.B. Dempsey (1976) J. Bacteriol. 126:166.

Willetts, N.S. and J. Maule (1974) Genet. Res. 24:81.

Willetts, N.S. and W. Paranchych (1974) J. Bacteriol. 120:101.

Wong, K. (1976) Ph.D. Thesis. University of Alberta, Edmonton.

Wong, K. and W. Paranchych (1976a) Virology 73:476.

Wong, K. and W. Paranchych (1976b) Virology 73:489.

Yamazaki, H. (1969) Virology 37:429.

APPENDIX A

Table for the Conversion of Refractive Index ($n^{25°}$) to Density in CsCl, SSC, pH 7.0 ($\rho^{5°}$)

$n^{25°}$	$\rho^{5°}$	$n^{25°}$	$\rho^{5°}$	$n^{25°}$	$\rho^{5°}$	$n^{25°}$	$\rho^{5°}$
1.3750	1.444	05	1.397	1.3660	1.349	15	1.302
49	1.443	04	1.396	59	1.348	14	1.301
48	1.442	03	1.395	58	1.347	13	1.300
47	1.441	02	1.394	57	1.346	12	1.299
46	1.440	01	1.393	56	1.345	11	1.298
45	1.439	1.3700	1.391	55	1.344	1.3610	1.297
44	1.438	99	1.390	54	1.343	09	1.296
43	1.437	98	1.389	53	1.342	08	1.295
42	1.436	97	1.388	52	1.341	07	1.294
41	1.435	96	1.387	51	1.340	06	1.293
1.3740	1.434	95	1.386	1.3650	1.339	05	1.292
39	1.433	94	1.385	49	1.338	04	1.291
38	1.432	93	1.384	48	1.337	03	1.290
37	1.431	92	1.383	47	1.336	02	1.289
36	1.430	91	1.382	46	1.335	01	1.288
35	1.428	1.3690	1.381	45	1.334	1.3600	1.286
34	1.427	89	1.380	44	1.333	99	1.285
33	1.426	88	1.379	43	1.332	98	1.284
32	1.425	87	1.378	42	1.331	97	1.283
31	1.424	86	1.376	41	1.330	96	1.282
1.3730	1.423	85	1.375	1.3640	1.328	95	1.281
29	1.422	84	1.374	39	1.327	94	1.280
28	1.421	83	1.373	38	1.326	93	1.279
27	1.420	82	1.372	37	1.325	92	1.278
26	1.419	81	1.371	36	1.324	91	1.277
25	1.417	1.3680	1.370	35	1.323	1.3590	1.275
24	1.416	79	1.369	34	1.322	89	1.274
23	1.415	78	1.368	33	1.321	88	1.273
22	1.414	77	1.367	32	1.320	87	1.272
21	1.413	76	1.366	31	1.319	86	1.271
1.3720	1.412	75	1.365	1.3630	1.318	85	1.270
19	1.411	74	1.364	29	1.317	84	1.269
18	1.410	73	1.363	28	1.316	83	1.268
17	1.409	72	1.362	27	1.315	82	1.267
16	1.408	71	1.361	26	1.314	81	1.266
15	1.407	1.3670	1.360	25	1.312	1.3580	1.265
14	1.406	69	1.359	24	1.311	79	1.264
13	1.405	68	1.358	23	1.310	78	1.263
12	1.404	67	1.357	22	1.309	77	1.262
11	1.403	66	1.356	21	1.308	76	1.261
1.3710	1.402	65	1.355	1.3620	1.307	75	1.260
09	1.401	64	1.354	19	1.306	74	1.259
08	1.400	63	1.353	18	1.305	73	1.258
07	1.399	62	1.352	17	1.304	72	1.257
06	1.398	61	1.351	16	1.303	71	1.256

$n^{25°}$	$\rho^{5°}$	$n^{25°}$	$\rho^{5°}$	$n^{25°}$	$\rho^{5°}$	$n^{25°}$	$\rho^{5°}$
1.3570	1.255	52	1.236	34	1.217	16	1.198
69	1.254	51	1.235	33	1.216	15	1.197
68	1.253	1.3550	1.233	32	1.215	14	1.196
67	1.252	49	1.232	31	1.214	13	1.195
66	1.251	48	1.231	1.3530	1.213	12	1.194
65	1.250	47	1.230	29	1.212	11	1.193
64	1.249	46	1.229	28	1.211	1.3510	1.192
63	1.248	45	1.228	27	1.210	09	1.191
62	1.247	44	1.227	26	1.209	08	1.190
61	1.246	43	1.226	25	1.208	07	1.189
1.3560	1.244	42	1.225	24	1.207	06	1.188
59	1.243	41	1.224	23	1.206	05	1.187
58	1.242	1.3540	1.223	22	1.205	04	1.186
57	1.241	39	1.222	21	1.204	03	1.185
56	1.240	38	1.221	1.3520	1.202	02	1.184
55	1.239	37	1.220	19	1.201	01	1.183
54	1.238	36	1.219	18	1.200	1.3500	1.182
53	1.237	35	1.218	17	1.199		

APPENDIX B

Table for the Conversion of Refractive Index ($n^{25°}$) to Density in Sucrose, SSC, ph 7.0 ($\rho^{5°}$)

$n^{25°}$	$\rho^{5°}$	$n^{25°}$	$\rho^{5°}$	$n^{25°}$	$\rho^{5°}$	$n^{25°}$	$\rho^{5°}$
1.450	1.372	1.421	1.276	1.392	1.182	1.363	1.087
1.449	1.369	1.420	1.273	1.391	1.178	1.362	1.084
1.448	1.366	1.419	1.267	1.390	1.175	1.361	1.080
1.447	1.362	1.418	1.267	1.389	1.171	1.360	1.077
1.446	1.359	1.417	1.264	1.388	1.168	1.359	1.074
1.445	1.356	1.416	1.260	1.387	1.165	1.358	1.071
1.444	1.353	1.415	1.257	1.386	1.161	1.357	1.068
1.443	1.350	1.414	1.254	1.385	1.158	1.356	1.064
1.442	1.346	1.413	1.251	1.384	1.155	1.355	1.061
1.441	1.343	1.412	1.248	1.383	1.152	1.354	1.057
1.440	1.339	1.411	1.244	1.382	1.149	1.353	1.054
1.439	1.336	1.410	1.241	1.381	1.145	1.352	1.051
1.438	1.332	1.409	1.237	1.380	1.142	1.351	1.047
1.437	1.329	1.408	1.234	1.379	1.139	1.350	1.044
1.436	1.326	1.407	1.231	1.378	1.136	1.349	1.041
1.435	1.322	1.406	1.227	1.377	1.133	1.348	1.038
1.434	1.319	1.405	1.224	1.376	1.129	1.347	1.035
1.433	1.316	1.404	1.221	1.375	1.126	1.346	1.031
1.432	1.312	1.403	1.218	1.374	1.123	1.345	1.028
1.431	1.309	1.402	1.215	1.373	1.120	1.344	1.025
1.430	1.306	1.401	1.211	1.372	1.117	1.343	1.022
1.429	1.303	1.400	1.208	1.371	1.113	1.342	1.019
1.428	1.300	1.399	1.204	1.370	1.110	1.341	1.015
1.427	1.296	1.398	1.201	1.369	1.106	1.340	1.012
1.426	1.293	1.397	1.198	1.368	1.103		
1.425	1.290	1.396	1.194	1.367	1.100		
1.424	1.286	1.395	1.191	1.366	1.096		
1.423	1.283	1.394	1.188	1.365	1.093		
1.422	1.280	1.393	1.185	1.364	1.090		

APPENDIX C

Papers Arising from this Thesis

Frost, L.S. and W. Paranchych (1977) Composition and Molecular Weight of Pili Purified from Pseudomonas aeruginosa K. J. Bacteriol. 131:259.

Frost, L.S., M. Carpenter and W. Paranchych (1978) N-Methylphenylalanine at the N-terminus of Pilin Isolated from Pseudomonas aeruginosa K. Nature (London) 271:87.

Paranchych, W., L.S. Frost and M. Carpenter (1978) The N-Terminal Amino Acid Sequence of Pilin Isolated from Pseudomonas aeruginosa K. J. Bacteriol. (in press).